Cellular Immunology 322 (2017) 64–73

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

Research paper

Piper nigrum extract ameliorated allergic inflammation through inhibiting Th2/Th17 responses and mast cells activation

Thi Tho Bui[a], Chun Hua Piao[a], Chang Ho Song[a,b], Hee Soon Shin[c,d], Dong-Hwa Shon[c,e], Ok Hee Chai[a,b,*]

[a] Department of Anatomy, Chonbuk National University Medical School, Jeonju, Jeonbuk 54896, Republic of Korea
[b] Institute for Medical Sciences, Chonbuk National University, Jeonju, Jeonbuk 54896, Republic of Korea
[c] Food Biotechnology Program, Korea University of Science and Technology, Daejeon 305-350, Republic of Korea
[d] Division of Nutrition and Metabolism Research, Korea Food Research Institute, Bundang-gu, Seongnam-si, Kyeonggi-do 463-746 Republic of Korea
[e] Division of Functional Food Research, Korea Food Research Institute, Bundang-gu, Seongnam-si, Kyeonggi-do 463-746, Republic of Korea

ARTICLE INFO

Keywords:
Piper nigrum
Allergic asthma
Mast cells
Th1/Treg
Th2/Th17
Anti-OVA specific IgE

ABSTRACT

Piper nigrum (Piperaceae) is commonly used as a spice and traditional medicine in many countries. *P. nigrum* has been reported to have anti-oxidant, anti-bacterial, anti-tumor, anti-mutagenic, anti-diabetic, and anti-inflammatory properties. However, the effect of *P. nigrum* on allergic asthma has not been known. This study investigated the effect of *P. nigrum* ethanol extracts (PNE) on airway inflammation in asthmatic mice model. In the ovalbumin (OVA)-induced allergic asthma model, we analysed the number of inflammatory cells and cytokines production in bronchoalveolar lavage fluid (BALF) and lung tissue; histological structure; as well as the total immunoglobulin (Ig)E, anti-OVA IgE, anti-OVA IgG_1 and histamine levels in serum. The oral administration (200 mg/kg) of PNE reduced the accumulation of inflammatory cells (eosinophils, neutrophils in BALF and mast cells in lung tissue); regulated the balance of the cytokines production of Th1, Th2, Th17 and Treg cells, specifically, inhibited the expressions of GATA3, IL-4, IL-6, IL-1β, RORγt, IL-17A, TNF-α and increased the secretions of IL-10, INF-γ in BALF and lung homogenate. Moreover, PNE suppressed the levels of total IgE, anti-OVA IgE, anti-OVA IgG_1 and histamine release in serum. The histological analysis showed that the fibrosis and infiltration of inflammatory cells were also ameliorated in PNE treated mice. On the other hand, PNE inhibited the allergic responses via inactivation of rat peritoneal mast cells degranulation. These results suggest that PNE has therapeutic potential for treating allergic asthma through inhibiting Th2/Th17 responses and mast cells activation.

1. Introduction

Allergic asthma has become an important public health problem globally. It is estimated that asthma affects 334 million people worldwide today and occurs in people of all ages. Asthma arise as a result of interaction between multiple genetic and environmental factors [1]. Pathologically, patients are found to have bronchial mucosal thickening by edema, bronchial wall remodeling, mucus overproduction, and eosinophil infiltration [2]. Recent advances in understanding the pathogenesis of allergic asthma have identified a complex interplay between T helper (Th) effector cells, recruited inflammatory eosinophils, local IgE-activated mast cells, and released inflammatory cytokines and chemokines, as well as mediators of airway spasm and hyper-reactivity [1,3–6]. Inhaled corticosteroids are currently the most effective drugs available for the treatment of asthma [7,8]. However, long-term use of inhaled steroids is often accompanied by undesirable adverse effects, particularly when high doses are used [9].

Piper nigrum, also known as black pepper, is a perennial woody evergreen climber native to South India that can grow to a height of 50–60 cm. It is an aromatic plant very well known for its antioxidant, antimicrobial, carminative, and antiseptic properties [10]. It has been used traditionally for the relief of pain, atrophic arthritis, apathy, influenza, and febricity, and as a nerve tonic, antibacterial agent, stimulant, digestive, antitoxin, and anti-inflammatory [11]. The essential oil of *P. nigrum* has been found to possess multiple applications in the food and pharmacological industries, perfumery, cosmetics and home

Abbreviations: PNE, *Piper nigrum* extract; Dex, Dexamethasone; OVA, ovalbumin; DMSO, dimethylsulfoxide; PBS, phosphate-buffered saline; BALF, bronchoalveolar lavage fluid; Ig, immunoglobulin; IL, interleukin; TNF-α, tumor necrosis factor alpha; INF-γ, interferon gamma. RORγt, Retinoic-related orphan receptor gamma t; GATA3, GATA binding protein 3

* Corresponding author at: Department of Anatomy and Institute for Medical Sciences, Chonbuk National University Medical School, Jeonju, Jeonbuk 54896, Republic of Korea.
E-mail address: okchai1004@jbnu.ac.kr (O.H. Chai).

http://dx.doi.org/10.1016/j.cellimm.2017.10.005
Received 4 May 2017; Received in revised form 28 July 2017; Accepted 13 October 2017
Available online 16 October 2017
0008-8749/

remedies [12]. A major alkaloid component from *P. nigrum* is piperine. Black pepper and piperine have been reported to enhance anti-inflammatory properties. Anti-inflammatory activities of *P. nigrum* have been demonstrated in rat models of carrageenan-induced rat paw edema, cotton pellet-induced granuloma, and a croton oil-induced granuloma pouch [13]. Piperine has anti-inflammatory effects on IL 1β-stimulated fibroblast-like synoviocytes in rat arthritis models [14], and inhibiting the production of prostaglandin E2 and nitric oxide in RAW264.7 cells [15]. Piperine showed an anti-inflammatory effect in S. *aureus*-induced endometritis via inhibiting the expression of TLR-2 and TLR-4 and the activation of the NFκB and MAPKs pathways, thereby preventing the excessive secretion of proinflammatory factors (TNF-α, IL-1β, and IL6) and increasing the secretion of an anti-inflammatory factor (IL-10) in mice models [16]. Nevertheless, anti-asthmatic effects of *P. nigrum* have been currently unclear. This study aims to investigate the effects of *P. nigrum* ethanol extract (PNE) on OVA-induced asthma mouse model.

In addition, we know that CD4+ T helper cells, one subgroup of lymphocytes, have an essential role in the immune system. T helper cells can differentiate into one of several subclasses, including Th1, Th2, Th17 and Treg which produce different cytokines and chemokines to promote a specific type of immune response [17,18,19]. Th1 induced by intracellular pathogens secrete predominantly interferon (IFN)-γ. Th2 cells are involved in host defence against extracellular pathogens and produce interleukin (IL) IL-1β, IL-4, IL-5, IL-6 as a result of the action of GATA3 and STAT6 transcription factors [20]. Th17 cells are characterized by their production of IL-17. Retinoid-related orphan receptor γt (RORγt) are required for Th17 cell differentiation [21]. Regulatory T cell (Treg) can inhibit various effector lymphocytes, including Th2, and Th17 [22]. Mast cells are associated with allergic diseases including allergic, asthma and autoimmune diseases [23]. Mast cells contain numerous granules including histamine, inflammatory cytokines, prostaglandins, and leukotrienes. Mast cells degranulation can be elicited by the basic secretagogues [24]. Compound 48/80 can increase the intracellular calcium content, which leads to histamine release from mast cells [25]. Compound 48/80 also causes a rapid release of inflammatory substances only from connective tissue-type mast cells, specifically rat peritoneal mast cells (RPMCs) [26]. In present study, we determined the effect of *P. nigrum* on allergic mechanisms via balance regulation of cytokine productions in differentiation T helper cells; specifically; Th1 (IFN-γ), Treg (IL-10), Th2 (transcription factor GATA3, IL-1β, IL-4, IL-6), Th17 (transcription factor RORγt, IL-17A) and TNF-α, also via prevention of rat peritoneal mast cells degranulation in *in vitro* study.

2. Materials and methods

2.1. Animals

Female BALB/c mice (6 weeks old) were purchased from the Damool Science (Dae-jeon, Korea). All procedures of this study were in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School (CBN 2013-0011) and were approved by the National Institutes of Health.

2.2. Asthmatic model establishment and treatment

Female BALB/c mice were divided into four groups (n = 6 each group), including control group (negative control, saline sensitization/challenge), OVA group (positive control, OVA sensitization/challenge), PNE group (OVA sensitization/challenge + 200 mg/kg PNE oral treatment) and Dex group (OVA sensitization/challenge + 2.5 mg/kg Dexamethasone oral treatment).

An OVA-induced asthma mouse model was established as described in Fig. 1. Briefly, mice were sensitized on day 1 with an intraperitoneal injection of 200 μL saline along with 50 μg OVA (Grade V, Sigma, St. Louis, MO, USA) and 1 mg aluminum hydroxide (Thermo Scientific, Rockford, MD, USA). On day 14, mice were sensitized 200 μL saline containing 50 μg OVA without aluminum hydroxide. After sensitization, on days 27, 28 and 29, mice received an airway challenge with OVA (5%, w/v, in saline) for 20 min using an ultrasonic nebulizer (NE-U17, Omron Co., Tokyo, Japan). Mice in the control group were sensitized and challenged with saline. On days 15–26, mice in the treatment groups were orally administered once daily with PNE and Dex at dose 200 mg/kg and 2.5 mg/kg, respectively; control group and OVA group were given saline. Mice were sacrificed 24h after the last OVA challenge.

2.3. Preparation of the PNE

PNE was provided by research grants from the Korea Food Research Institute and managed with specimen number (KFRI-SL-1105). Black pepper was purchased from a rural market (Gyeonggi-do, Korea). The extract was prepared using a soxhlet flux extractor. Briefly, the black pepper was incubated in 70% ethanol at 65 °C for two periods of 3 h each. The 70% ethanol extract was filtered using filter paper (Whatman No. 4), before the extract was concentrated by rotary evaporator (IKA RV10, USA) and dried in a freeze dryer. The dried black pepper extract (PNE) was stored at 4 °C until used. DMSO, widely used as a solvent for drugs testing in asthma. The previous study showed that 10% DMSO had no significant mobilization of eosinophils and neutrophils into the lungs, also no antioxidative effects in an allergic asthma mouse model when compared to saline-instilled mice [27]. Thus, 0.1% concentration of DMSO used as a solvent of PNE in our study. For asthmatic mice model treatment, PNE (200 mg/kg) was dissolved in 0.1% dimethylsulfoxide (DMSO) (Sigma, France) in saline and was prepared fresh daily before each treatment. For *in vitro* test, PNE dose-dependently (0.1, 1 and 10 mg/mL) was dissolved in saline.

2.4. Bronchoalveolar lavage fluid collection and cell count

Mice were sacrificed one day after the final challenge. BALF was collected by flushing 1mL of ice-cold PBS into the trachea and withdrawn through the cannula. BALF was centrifuged at 1000 RPM/min for 10 min at 4 °C. The supernatant was stored at −70 °C for ELISA analysis. The cell pellets were resuspended in 0.7 mL PBS and the total cell numbers were counted in a hemocytometer.

To determine differential cell counts, 150 μL of BALF was centrifuged onto slides using a cytospin device (Hanil Science Industrial, Korea) (1000 rpm, 10 min, 4 °C). Diff-Quik Staining reagent (1-5-1 Wakinohama-Kaigandori, Chuo-Ku, Kobe, Japan) was used for the cell staining according to the protocols from the manufacturer.

2.5. Histological examination of lung tissues

After BALF was obtained, the middle lobe of the lung was removed and fixed in 10% (v/v) neutral buffered formalin. Tissues were embedded in paraffin, sectioned at 4 μm thickness. The lung tissues were stained with Hematoxylin and Eosin (H & E) (Sigma, St. Louis, MO), Congo Red (Sigma, St. Louis, MO), Toluidine Blue (Sigma, St. Louis, MO), Periodic Acid-Schiff (PAS) (Sigma, St. Louis, MO) and Masson Trichrome (Sigma, St. Louis, MO) to assess structure of the lung, inflammatory cell infiltration, mast cell infiltration, mucus production and collagen deposition, respectively.

H & E staining of lung tissues was observed under a light microscope for histopathological examination. Lung injury was quantified according to the methods provided by Bello et al. [28]. Each slide was evaluated by two separate investigators. A total of 300 alveoli were counted on each slide at x400 magnification. The lung was assessed for alveolar congestion, alveolar hemorrhage, intra-alveolar fibrin and intra-alveolar infiltrates of cells. The injury score was calculated according to the formula: injury score = [(alveolar hemorrhage points/

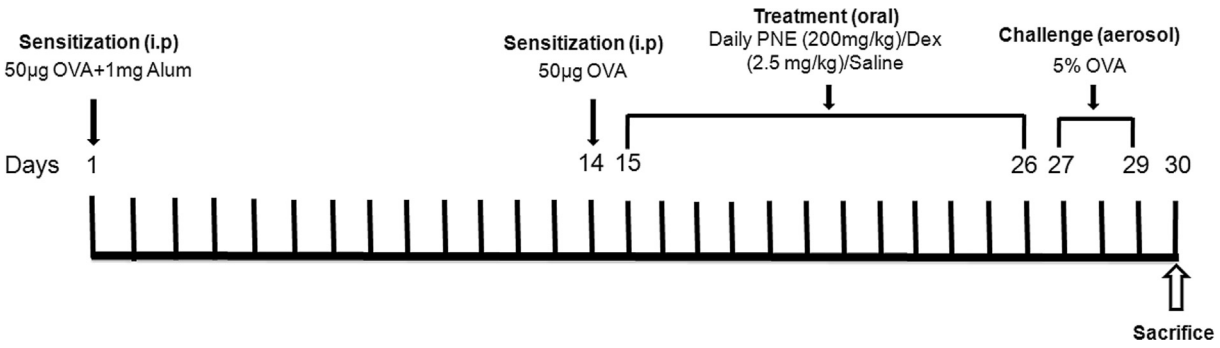

Fig. 1. Protocol for allergic asthma mouse model and treatment with PNE. Mice were sensitized on days 1 and 14, and challenged on days 27, 28 and 29 by OVA. Mice in control group were sensitized and challenged by saline. Mice in the PNE or Dex treatment group were administered orally once a day at 200 mg/kg PNE or 2.5 mg/kg Dex for 12 days.

No. of fields) + 2 × (alveolar infiltrate points/No. of fields) + 3 × (fibrin points/No. of fields) + (alveolar septal congestion/No. of fields)]/ total number of alveoli counted.

2.6. Measurement of the differentiation helper T cells productions in BALF and lung homogenate

Lung tissue was homogenized (Omni Prep Rotor Stator Generator, Omni International, USA) and centrifuged to obtain supernatant. The BALF was centrifuged to collected the supernatant. The concentration of Th1 production (INF-γ), Treg production (IL-10), Th2 productions (GATA3, IL-1β, IL-4, IL-6), Th17 productions (RORγt, IL-17A) and TNF-α were examined by using ELISA (R & D Systems Inc. USA) according to the protocols from the manufacturer.

2.7. Measurement of the total IgE, anti-OVA-specific IgE, anti-OVA-specific IgG_1 and histamine release in serum

Blood was collected 24h after the last challenge from mice and serum was obtained by centrifugation (1000 rpm, 10 min, 4 °C) and stored at −70 °C. The levels of total immunoglobulin E (IgE), anti-OVA-specific IgE, anti-OVA-specific IgG_1 and histamine were quantified using ELISA kits (R & D Systems Inc. USA) according to the manufacturers' instruction.

2.8. Compound 48/80-induced rat peritoneal mast cells (RPMCs) degranulation

RPMCs were isolated as previously described [29] and purified by using a Percoll density gradient as described in detail elsewhere [30]. Purified RPMCs (1×10^6 cells/mL) were resuspended in HEPES Tyrode buffer (136 mM NaCl, 5 mM KCl, 2 nM $CaCl_2$, 11 mM $NaHCO_3$, 0.6 mM NaH_2PO_4, 2.75 mM $MgCl_2$, 5.4 mM HEPES, 1.0 mg/mL BSA, 1.0 mg/mL glucose, 0.1 mg/mL heparin, pH 7.4). RPMCs (1×10^6 cells/200μL) were preincubated with 25 μl PNE (10, 1, 0.1 mg/mL) or saline for 10 min at 37 °C and then incubated with 25 μl compound 48/80 (5 μg/mL) or saline for 15 min. After incubation, the RPMCs were observed under microscope to photograph and analysis the percentage (%) of mast cells degranulation.

2.9. Statistical analysis

The results were performed by using Graph Pad Prism 5 (La Jolla, CA, USA). Data were expressed as means ± SEM. The differences among the groups were analyzed with one-way ANOVAs followed by Tukey's test. Statistical significance was considered at $P < 0.05$.

3. Results

3.1. PNE reduced the recruitment of inflammatory cells in BALF

To examinate the effect of PNE on the airway inflammation, the number of total cell and differential inflammatory cells in BALF were measured. The number of total cell and eosinophils in BALF of OVA-challenged mice were significantly elevated compared with the control mice. By contrast, PNE treatment (200 mg/kg) significantly reduced the increase of these cells in BALF when compared to OVA group ($P < 0.001$). Although the neutrophils and macrophages number showed tendency to decrease in the PNE group, there was no statistically significant difference ($P > 0.05$) (Fig. 2A). The Diff-Quick stain showed the present of differential inflammation cells in BALF and black arrows indicated as eosinophils and neutrophils infiltration. The eosinophils and neutrophils numbers were clearly decreased in BALF of PNE-treated mice (Fig. 2B).

3.2. PNE reduced histopathological changes in lung tissue

For the mice in the asthmatic group, the bronchus showed significantly changes compared with the control mice, specifically, H & E staining indicated that the airway epithelium was edema and the smooth muscle layer was thickened (Fig. 3B1). However, for the PNE (200 mg/kg) treatment group, airway epithelium and smooth muscles were thinner than those in the asthmatic mice (Fig. 3C1). Congo red staining indicated that asthmatic mice showed the infiltration of eosinophils in peribronchial and alveolar septal regions (Fig. 3B2). However, treatment with PNE considerable decreased the eosinophils infiltration compared with the asthmatic mice (Fig. 3C2). Mucus overproduction in the asthmatic mice was observed as a violet color in the bronchial airways compared with the control group (Fig. 3B3). In contrast, administration of PNE significantly suppressed the mucus hypersecretion in lung tissue compared with that observed in the asthmatic group (Fig. 3C3). Masson trichrome staining showed that PNE-treated mice were inhibited collagen deposition in peribronchial and perivascular (Fig. 3C4) when compared with the asthmatic mice (Fig. 3B4). Toluidine blue staining indicated mast cells infiltration in the lung tissue which was observed as dark blue color. The mast cells number were higher in asthmatic mice (Fig. 3B5) compared to PNE-treated mice (Fig. 3C5). The histopathological changes on asthmatic mice model also attenuated by treatment with Dex (Fig. 3D1-D5). In addition, the category for lung injury examination including alveolar congestion, alveolar hemorrhage, intra-alveolar fibrin and intra-alveolar infiltrates were also assessed for quantifying lung injury. The higher the histological score, the greater severity of the injury. Histological score was markedly increased in OVA group and decreased in PNE and Dex treatment (Fig.3E).

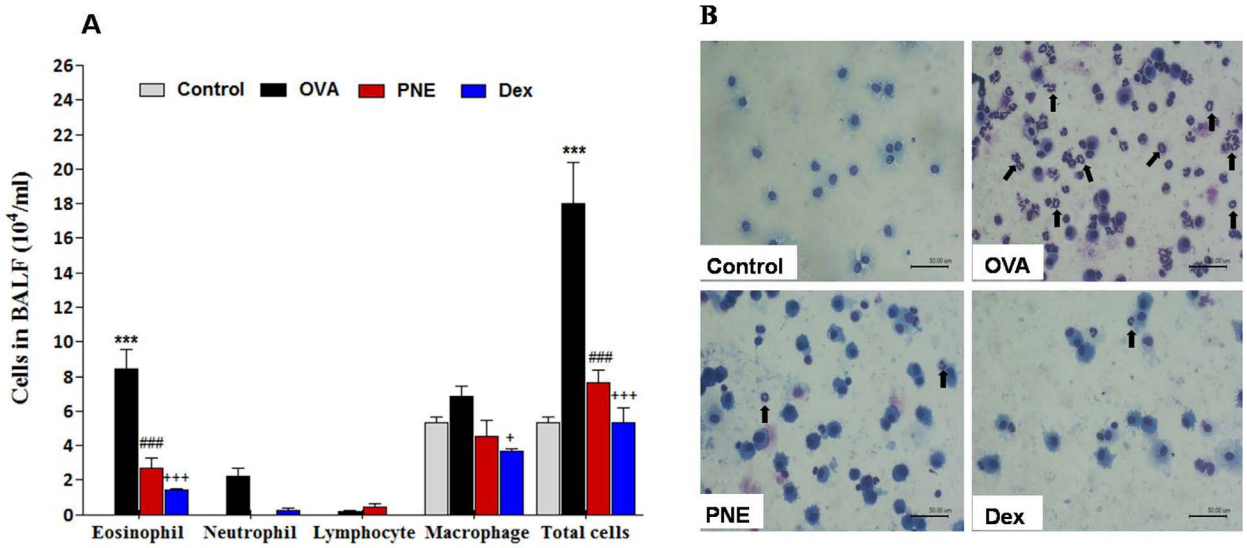

Fig. 2. PNE reduced the number of differential cells and total cells in BALF (A) and inhibited the recruitment of inflammatory cells in BALF (B). Control group: saline challenge. OVA group: OVA challenge. PNE: OVA challenge, PNE treatment (200 mg/kg). Dex: OVA challenge, Dexamethasone treatment (2.5 mg/kg). (A) The values represent the mean ± S.E.M of three independent experiments (n = 6/group). Significant differences at $^{***}P < 0.001$ compared with the Control group. $^{\#\#\#}P < 0.01$ compared with the OVA-induced group. $^{+++}P < 0.001$ and $^{+}P < 0.05$ compared with the PNE group. (B) BALF was collected 24h after the last OVA challenge, the cell were isolated by cytospin and stained with Diff-Quick. Black arrows indicated as eosinophils and neutrophils. Scale bar: 50 μm.

3.3. PNE regulated the balance of helper T cells responses in the BALF and lung homogenate

We evaluated the balance of Th-related productions in BALF and lung homogenate to investigate the effect of PNE on asthmatic mice. In the OVA-induced mice; the levels of IL-1β, IL-4, IL-6 cytokines, GATA3 transcription factor (Th2); IL-17A cytokine, RORγt transcription factor (Th17) and TNF-α cytokine were significantly increased in both BALF and lung homogenate, while IFN-γ (Th1) did not change the level in BALF and lung homogenate. IL-10 (Treg) even reduced in lung homogenate compared to control group. By contrast; PNE-treated mice resulted in a markedly decrease the inflammatory productions as GATA3, IL-1β, IL-4 (Th2); RORγt, IL-17A (Th17) and TNF-α. Except for IL-6 (Th2) cytokine only significantly decreased in BALF, there had a tendence to decrease in lung tissue compared to the OVA group but no statistically significant difference. Meanwhile, PNE treatment was elevated IFN-γ (Th1) and IL-10 (Treg) productions in BALF and lung homogenate when compared with those in the OVA-induced mice (Fig. 4).

3.4. PNE reduced the levels of IgE, anti-OVA specific IgE and anti-OVA specific IgG_1 in serum

Because Th2 cytokines play important role on the allergic responses via the regulation in IgE, anti-OVA specific IgE and anti-OVA specific IgG_1 levels, we evaluated their expression in the serum. The level of total IgE, anti-OVA specific IgE and anti-OVA specific IgG_1 were significantly up-regulated in OVA-induced mice compared with those in the control mice. However, PNE treatment (200 mg/kg) led to a decrease levels of total IgE, anti-OVA specific IgE and anti-OVA IgG_1 in the serum compared with the OVA group (Fig. 5).

3.5. PNE inhibited mast cells infiltration into lung and histamine release into serum

To examine the effects of PNE on the role of mast cells in allergic mechanisms, we determined the expression of mast cells number in the lung tissue and histamine release in serum of OVA-induced asthmatic mice. Mast cells number significantly elevated in asthmatic mice and markedly reduced with PNE and Dex treatment. It is well known that activated mast cells can produce histamine. The percentage of mast cells degranulation was significantly higher in asthmatic mice and lower in PNE and Dex treatment. Accordingly, the histamine release in serum also strongly increased in asthmatic mice and decreased in PNE and Dex treatment (Fig. 6).

3.6. PNE prevented compound 48/80-induced RPMCs degranulation

To determine the direct effect of PNE on mast cells that mediated allergic responses, we observed the degranulation of RPMCs under the microscope. Fig. 7A showed that the normal RPMCs were oval, spindle, or polygonal in shape and had many closely packed secretory granules. When RPMCs were incubated with PNE (10, 1, 0.1 mg/mL) alone, there were similar finding as seen in normal RPMCs. However, after stimulation with compound 48/80 for 15 min, RPMCs were clearly showed extensive degranulation processes and the presence of swelling, cytoplasmic vacuoles, and multiple granules extruding from the cells surface. On the contrary, PNE treatment at doses of 10, 1, 0.1 mg/mL for 10 min prior to compound 48/80 stimulation were suppressed the degranulation and RPMCs morphology was similar to that of normal cells. Fig. 7B showed that, in compound 48/80-treated RPMCs, the percentage of mast cells degranulation significantly increased as compared to control group. However, PNE dose-dependently (10, 1, 0.1 mg/mL) significantly inhibited the compound 48/80-induced the mast cells degranulation.

4. Discussion

It is well known that inflammatory responses may be prevented by inhibiting excess production of cytokines from Th2, Th17 cells. Previous study showed that the Th2 cell responses associated with inflammation, including dominant IgG_1, elevate serum IgE, increase Th2 cytokines, and eosinophils infiltration [31]. Th1 and Th2 productions have the interaction in the allergy responses. An imbalance between Th1 and Th2 cells leads to the clinical expression of allergic disease [32]. On the other hand, Th17 cells have been associated with Th2-predominant allergic diseases and showed to play proinflammatory roles in the development of asthma and chronic inflammation [33,34]. Treg cell can inhibit Th2, and Th17 responses [22]. The Treg/Th17 imbalance has an important role in induction of inflammatory immune

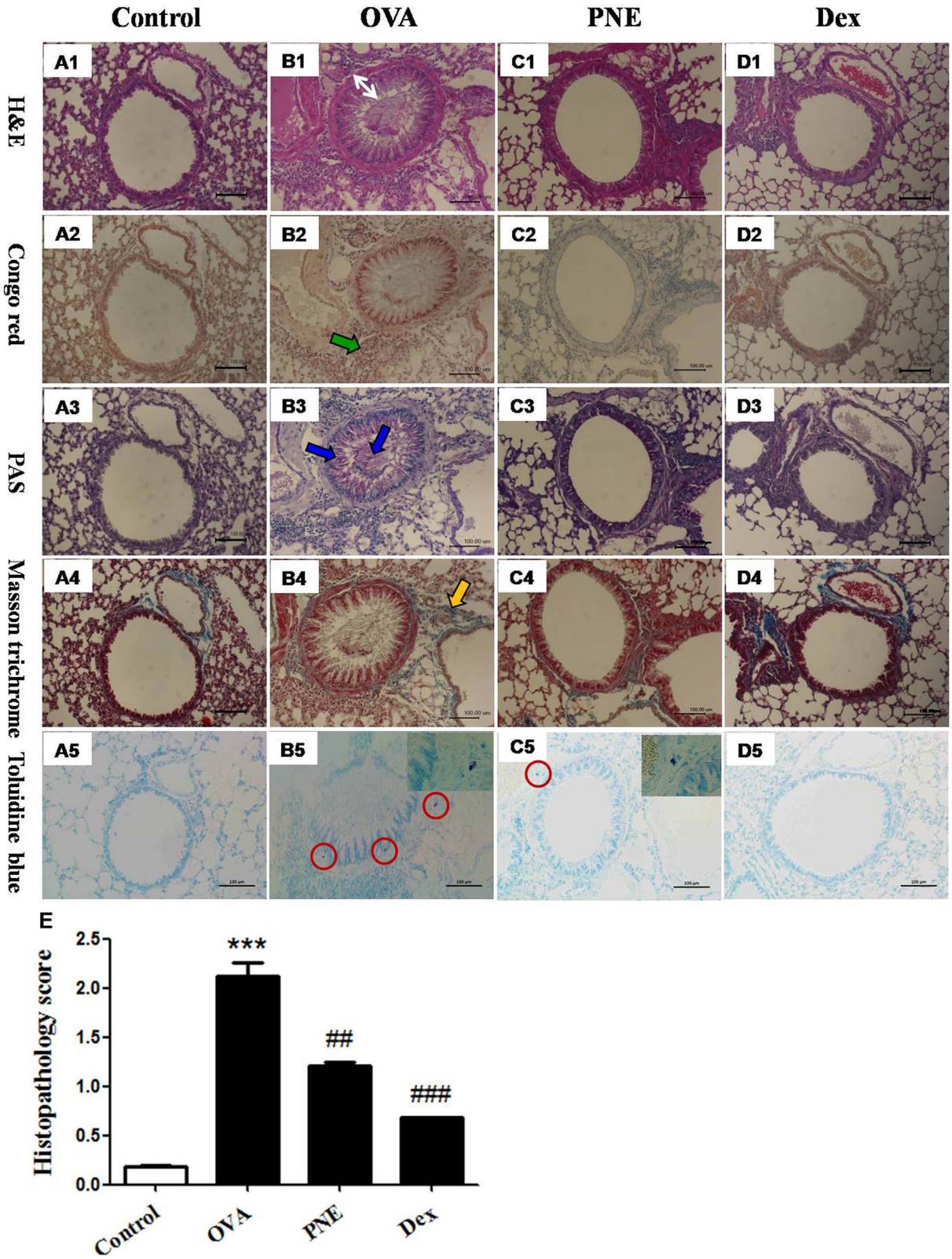

Fig. 3. PNE prevented histological changes of the lung tissue in OVA-induced asthma model. (A1–A5) Control group, (B1–B5) OVA group, (C1–C5) PNE group: OVA challenge, 200 mg/kg PNE. (D1–D5) Dex group: OVA challenge, 2.5 mg/kg Dexamethasone. The sections were stained with H & E, Congo Red, PAS, Masson Trichrome and Toluidine Blue. White arrow indicated the bronchial-wall thickness. Green arrow indicated eosinophils infiltration. Blue arrows indicated mucus hypersecretion (violet color in the bronchial airways). Yellow arrow indicated as collagen fiber deposits (blue color in peribronchial and perivascular). The red circle indicated mast cells infiltration in the lung tissue. Scale bar: 100 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

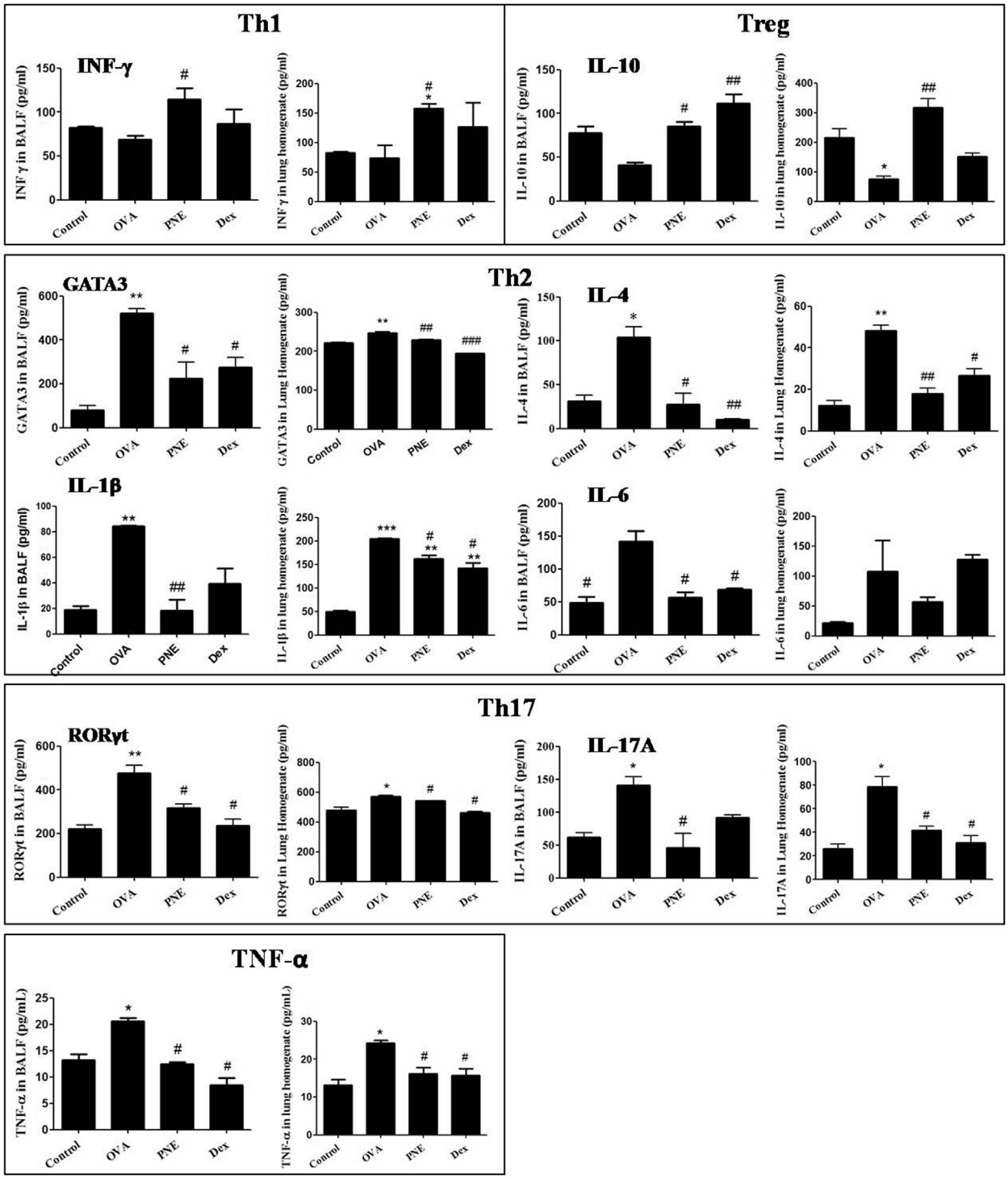

Fig. 4. PNE improved the imbalance of the differentiation helper T cells responses in BALF and lung homogenate. The concentration of Th1 production (INF-γ), Treg production (IL-10), Th2 productions (GATA3, IL-1β, IL-4, IL-6), Th17 productions (RORγt, IL-17A) and TNF-α in BALF and lung homogenate were determined by ELISA kits. Control group: saline challenge. OVA group: OVA challenge. PNE: OVA challenge, PNE treatment (200 mg/kg). Dex: OVA challenge, Dexamethasone treatment (2.5 mg/kg). The values represent the mean ± S.E.M of three independent experiments (n = 6/group). Significant differences at $^{***}P < 0.001$, $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with the control group. $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared with the OVA-induced group.

diseases [35].

In present study, we investigated the anti-asthmatic activities of PNE using OVA-induced asthma mice model. Allergic airway disease is a complex condition characterized by increased amounts of systemic IgE, elevated allergen-specific T helper 2 cells and their products, airway hyperreactivity (AHR), and structural changes in the lung [36]. The inflammatory process may be divided into early- and late-phase reactions. The early (immediate) response is usually mediated by mast cell degranulation, whereas late phase is followed by neutrophil, eosinophil, and lymphocyte migration to the inflammatory site [1].

Our data shown that OVA-induced mice elevated number of inflammatory cells; increased total IgE, anti-OVA specific IgE and anti-OVA specific IgG_1 productions; imbalance of Th1/Th2, Treg/Th17 in BALF and lung homogenate; mucus over production; eosinophil-rich

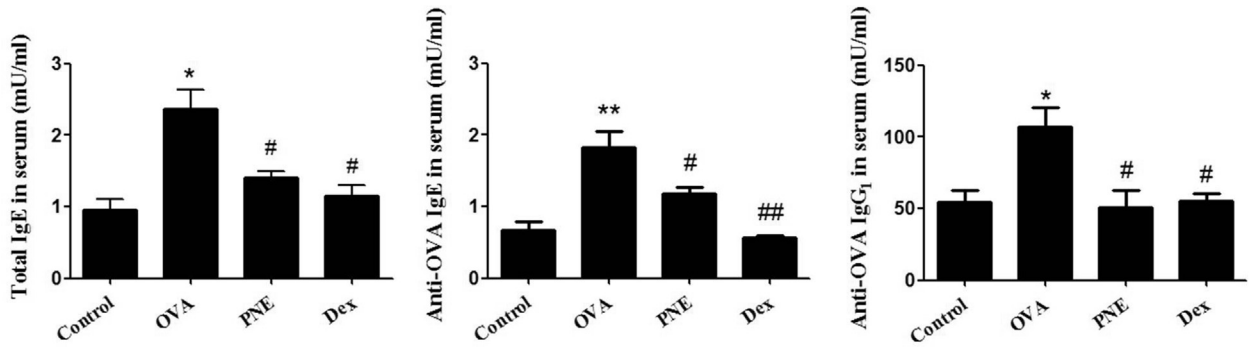

Fig. 5. PNE reduced the levels of total IgE, anti-OVA specific IgE and anti-OVA specific IgG_1 in the serum. Control group: saline challenge. OVA group: OVA challenge. PNE: OVA challenge, PNE treatment (200 mg/kg). Dex: OVA challenge, Dexamethasone treatment (2.5 mg/kg). The values represent the mean ± S.E.M of three independent experiments (n = 6/group). Significant differences at $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with the control group. $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared with the OVA-induced group.

leukocyte infiltration. However, PNE treatment resulted in an elevation the productions of Th1 (INF-γ), Treg (IL-10) and reduction the productions of Th2 (GATA3, IL-1β, IL-4, IL-6), Th17 (RORγt, IL-17A) and TNF-α as well as downregulation total IgE, anti-OVA specific IgE, anti-OVA specific IgG_1 and histamine level in serum. The number of inflammatory cells infiltration in BALF, mucus hypersecretion was also clearly inhibited. Accordingly, this implies that PNE have anti-inflammation and anti-asthmatic effects on OVA-induced airway inflammation in an asthma mice model.

The infiltration of eosinophils into the airway is a characteristic of asthma, and increased numbers of these cells have been found in BALF. The proinflammatory mediators derived from eosinophils are major contributors to the inflammation observed in asthma, which includes airway epithelial cell loss and damage, airway dysfunction, and mucus hypersecretion [37]. In addition, mucus hypersecretion, an important feature of asthma, is induced by eosinophils and mast cells products [38]. We found that oral treatment of PNE significantly decreased the number of eosinophil and neutrophil in BALF. The results of histopathological examination was also paralleled the cell count in BALF. Histopathological has been shown that PNE treatment markedly suppressed the infiltration of inflammation cells and mucus over production of goblet cells in lung tissues. Furthermore, scoring lung injury in control mice showed that the alveolar structure was delicate and clear. In the contrast, the alveolar congestion, alveolar hemorrhage, intra-alveolar fibrin and intra-alveolar inflammatory cells were observed in OVA group with high values of histological score. After PNE treatment, the score of lung injury was markedly reduced, indicating lung injury was significantly attenuated.

An imbalance between Th1 and Th2 responses lead to excessive Th1 cells or Th2 cells activation. Allergic diseases are characterized by a predominant Th2 response [39]. Previous studies have shown that modulation of Th1/Th2 cytokines can inhibit airway inflammation in OVA-induced mice [40,41]. Among the cytokines released from Th1, Th2 cells, IFN-γ has been reported to inhibit the synthesis of IgE and the differentiation of precursor cells to Th2 cells, increased levels of IFN-γ have been used to explain the anti-allergic effects of therapeutic agents [42]. IFN-γ also play an important inhibitory role on recruitment of eosinophils to the lung [43]. On the other hand, IL-6 has emerged as an important regulator of effector CD4 T cell fate, promoting IL-4 production during Th2 differentiation, inhibiting Th1 differentiation [44]. Our data showed that the OVA induced mice was higher Th2 productions (transcription factor GATA3, IL-4, IL-6) and lower Th1 cytokine levels (INF-γ) compared with those in the control group. In the contrast, PNE treatment downregulated Th2 productions (IL-6 in BALF; GATA3, IL-4 in BALF and lung tissue) and upregulated Th1 cytokine (IFN-γ) in both BALF and lung homogenate compared with OVA group, demonstrating the effect of PNE on the balance of Th1/Th2, therefore, showing the anti-allergic effects of PNE.

In addition, an approach to suppress Th17 cell differentiation and regulate Treg/Th17 imbalances could be an effective treatment for chronic inflammatory diseases [35]. IL-10 is an inhibitory cytokine of inflammation and revealed to be produced by Th1 and Tregs [45,46]. Because of its immunosuppressive properties *in vitro* and in animal models, IL-10 has been suggested as a potential therapy of allergic inflammation and asthma [47]. Our data showed that IL-10 from BALF and lung homogenate significantly increased, meanwhile, transcription factor RORγt and IL-17A was decreased, indicating the effect of PNE on the regulation T cells and Th17. These findings suggest that PNE could attenuate the development of allergic inflammation via regulating the balance of Treg/Th17 cytokines.

The levels of IL-6 in serum have been found to be elevated in a number of inflammatory diseases. IL-6 has long been considered a general marker of inflammation together with TNF-α and IL-1β, two other classical inflammatory cytokines [48]. IL-6 and TNF-α directly or indirectly play important roles in the recruitment and activation of inflammatory cells [49,50]. TNF-α is a chemotactic cytokine for

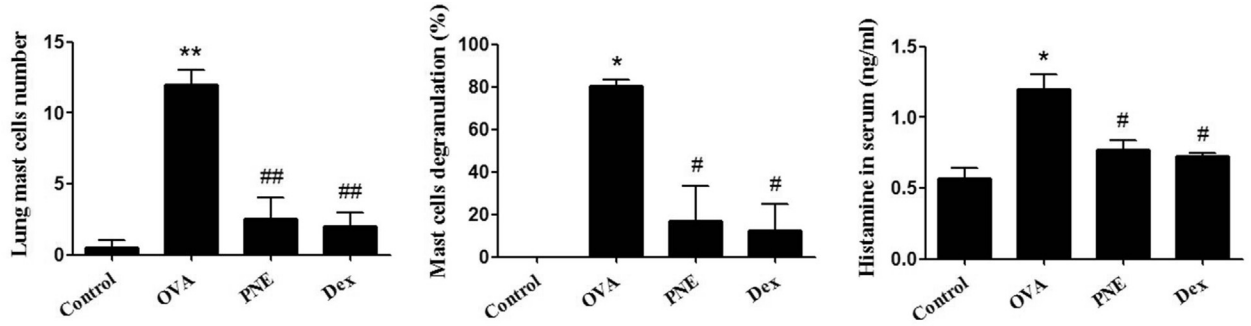

Fig. 6. PNE inhibited mast cells infiltration in lung tissue and the histamine level in serum. Control group: saline challenge. OVA group: OVA challenge. PNE: OVA challenge, PNE treatment (200 mg/kg). Dex: OVA challenge, Dexamethasone treatment (2.5 mg/kg). The values represent the mean ± S.E.M of three independent experiments (n = 6/group). Significant differences at $^{**}P < 0.01$ and $^{*}P < 0.05$ compared with the control group. $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared with the OVA-induced group.

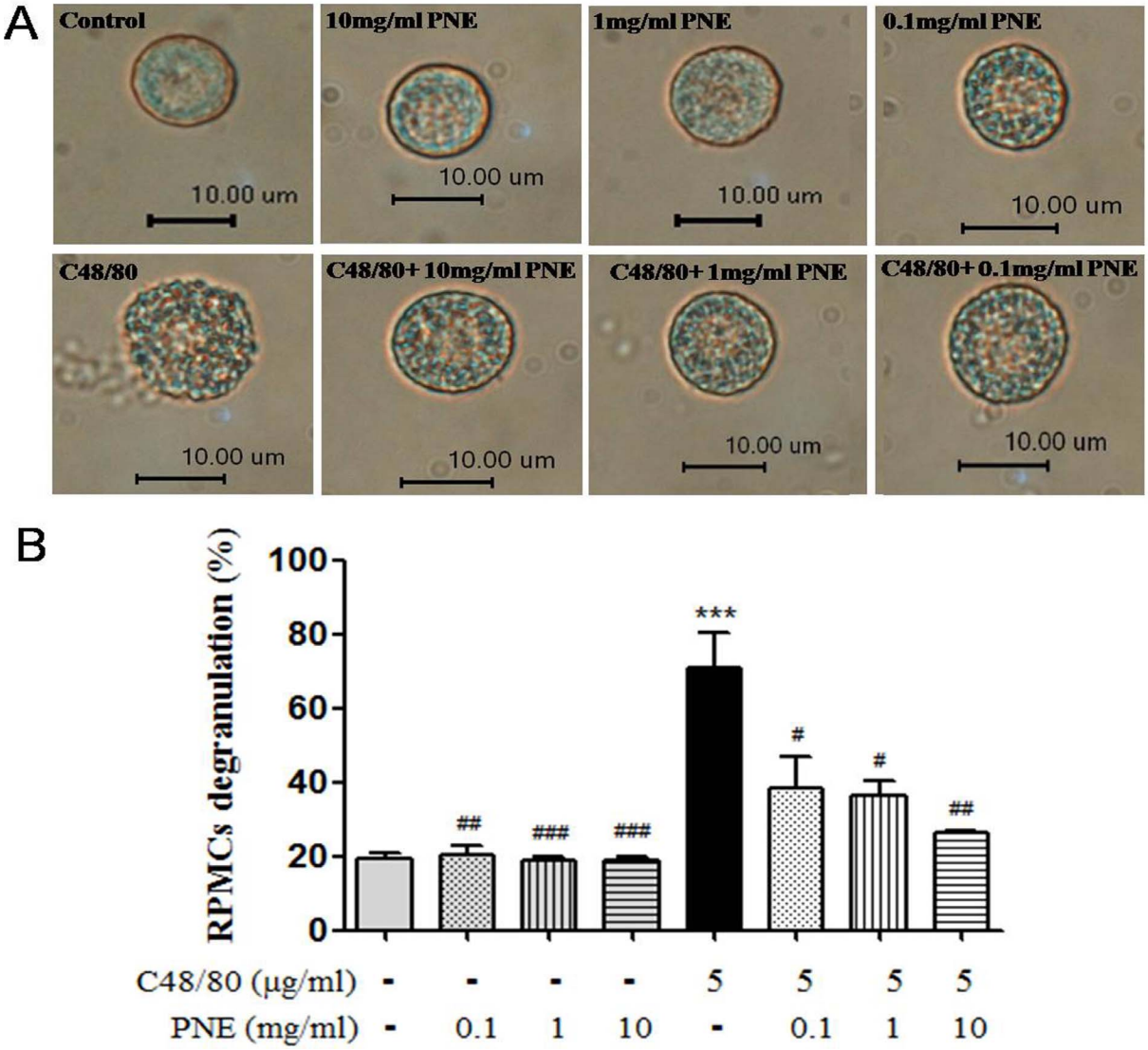

Fig. 7. PNE prevented compound (C) 48/80-induced rat peritoneal mast cells (RPMCs) degranulation. RPMCs (1×10^6 cells/200 μL) were pretreated with 25 μl PNE (10, 1, 0.1 mg/mL) or saline for 10 min at 37 °C and then incubated with 25 μl C48/80 (5 μg/mL) or saline for 15 min. PNE dose-dependently inhibited the C48/80-induced RPMCs degranulation. (A) Inverted light microscopy of RPMCs, bar = 10μm. (B) Percentage (%) of RPMCs degranulation. The values represent the mean ± S.E.M of three independent experiments (n = 6/group). Significant differences at $^{***}P < 0.001$ compared with the control group. $^{\#\#\#}P < 0.001$, $^{\#\#}P < 0.01$ and $^{\#}P < 0.05$ compared with the C48/80 group.

granulocytes including eosinophils and neutrophils [51]. Furthermore, *in vivo* and *in vitro* assays have demonstrated that IL-1β can alter airway function by inducing cellular infiltrate, airway eosinophilia, mucus hyperplasia, airway wall thickening, fibrosis, collagen synthesis, IgE synthesis, and enlargement of distal air spaces [52–55]. In support of these results, our findings showed that TNF-α, IL-6 and IL-1β were found to increase in BALF and lung homogenate in the OVA-induced mice. Meanwhile, PNE treatment led to a decreased of TNF-α, IL-6 and IL-1β levels in the BALF and lung homogenate when compared to those in the OVA group. Therefore, it is possible that the inhibition of airway wall thickening, mucus hypersecretion, collagen deposition, and inflammatory cells infiltration, especially, eosinophil result from reduction of TNF-α, IL-1β and IL-6 after oral treatment of PNE.

IgE, which is a key target for the development of anti-asthma strategies, is one of the most important factors in the progression of allergic reactions [56]. OVA-specific IgG in rat-sera, such as IgG_1 and IgG_{2a} increase on day 21 after OVA inhalation in asthmatic models induced by OVA [57]. Anti-OVA IgG_1 directly binds with FcgRIIB on DCs during allergic airway inflammation [58]. In present study, PNE treatment significantly reduced the overexpression of serum total IgE, anti-OVA specific IgE and anti-OVA specific IgG_1 in OVA-induced mice model, showing the effect of PNE on the inhibition development of allergic reactions. This finding is also consistent with the upregulation of Th1 (IFN-γ), Treg (IL-10) cytokines and the downregulation of Th2 (GATA3, IL-1β, IL-4, IL-6) and Th17 (RORγt, IL-17A) productions in BALF and lung homogenate.

All the mast cell-mediated inflammatory reactions are characterized by an accumulation of mast cell in the inflammatory sites [25]. In this study, the high infiltration of mast cells degranulation number was observed in peribronchial and other sites of lung tissue in asthmatic mice, meanwhile, PNE significantly reduced the mast cells accumulation as well as mast cells degranulation. Histamine, a major component of mast cell granules, exerts many effects related to the immediate-phase of allergic inflammation such as tissue edema, contraction of bronchial and smooth muscle, and increased mucus production [59]. We have been observed above features which were appeared in asthmatic mice and improved in PNE treatment along with the decrease of histamine level. Thus, these results showed that the decrease of histamine level maybe contributed to inhibit allergic symptoms. Histopathological features also shown that PNE treatment markedly reduced

the infiltration of mast cells in lung tissues, consistent with the decrease of histamine levels in serum. Therefore, these results provide the evidence for the anti-allergic effects of PNE via inhibition of mast cells degranulation which prevent the histamine release.

To investigate whether PNE mediated on mast cell allergic responses. We examined the inhibition of PNE on mast cell activation *in vitro* and the degranulation of RPMCs was observed. These results demonstrated that compound 48/80-induced RPMC degranulation was markedly attenuated by the PNE in the allergic response. Our study provide evidence that PNE have a strong effect in the prevention or treatment of mast cell degranulation, that led to may have suppresses the mast cell-mediated production release. The significant decrease of histamine level in PNE-treated asthmatic mice was correlated to these results, suggesting that PNE could reduce histamine release which is an essential production of allergic symptoms via inhibiting mast cell degranulation in allergic immune response.

In conclusion, the oral administration of PNE significantly inhibited airway inflammation in OVA-induced mouse model by reducing inflammation cells such as eosinophil, neutrophil, goblet cells, mast cells; decreasing of mucus hypersecretion; ameliorating collagen deposition in lung tissue; regulating the balance of Th1/Th2 cytokines and Treg/Th17 cytokines; attenuating expression of serum total IgE, anti-OVA IgE, anti-OVA IgG_1 and histamine levels. Moreover, PNE inhibited the allergic responses via inactivation of mast cells degranulation. The results obtained in this study suggest that PNE may be provide a promising strategy for immunotherapy such as allergic asthma treatment.

Conflict of interest

These authors declare no conflict of interest.

Acknowledgment

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2012R1A1A303857) and by research grants (E0121304-05) from the Korea Food Research Institute.

References

[1] GAR Global Asthma Report 2014 Available Online at: , 2014 (accessed 03.01.16).
[2] P.O. Girodet, A. Ozier, I. Bara, J.M. de Tunon Lara, R. Marthan, P. Berger, Airway remodeling in asthma: new mechanisms and potential for pharmacological intervention, Pharmacol. Ther. 130 (3) (2011) 325–337.
[3] L. Cohn, J.A. Elias, G.L. Chupp, Asthma: mechanisms of disease persistence and progression, Annu. Rev. Immunol. 22 (2004) 789–815.
[4] P.S. Foster, A.W. Mould, M. Yang, J. Mackenzie, J. Mattes, S.P. Hogan, S. Mahalingam, Elemental signals regulating eosinophil accumulation in the lung, Immunol. Rev. 179 (2001) 173–181.
[5] C.A. Herrick, K. Bottomly, To respond or not to respond: T cells in allergic asthma, Nat. Rev. Immunol. 3 (2003) 405–412.
[6] D.T. Umetsu, J.J. McIntire, O. Akbari, C. Macaubas, R.H. DeKruyff, Asthma: an epidemic of dysregulated immunity, Nat. Immunol. 3 (2002) 715–720.
[7] G. Caramori, K. Ito, I.M. Adcock, Targeting Th2 cells in asthmatic airways, Curr. Drug Targets Inflamm. Allergy 3 (3) (2004) 243–255.
[8] L.M. Tourangeau, A. Kavanaugh, S.I. Wasserman, The role of monoclonal antibodies in the treatment of severe asthma, Ther. Adv. Respir. Dis. 5 (3) (2011) 183–194.
[9] B.J. Lipworth, C.M. Jackson, Safety of inhaled and intranasal corticosteroids Lessons for the new millennium, Drug Saf. 23 (1) (2000) 11–33.
[10] S. Chatterjee, Z. Niaz, S. Gautam, S. Adhikari, P.S. Variyar, A. Sharma, Antioxidant activity of some phenolic constituents from green pepper (*Piper nigrum* L.) and fresh nutmeg mace (*Myristica fragrans*), Food Chem. 101 (2007) 515–523.
[11] T.J. Zachariah, V.A. Parthasarathy, Black Pepper, Chemistry of Spices, Oxford University Press, New York, 2008, pp. 21–40.
[12] P.N. Ravindran, J.A. Kallupurackal, Black Pepper, in: K. Peter (Ed.), Handbook of Herbs and Spices, CRC Press, Taylor & Francis Group, New York, 2004, pp. 62–110.
[13] A.M. Mujumdar, J.N. Dhuley, V.K. Deshmukh, P.H. Raman, S.R. Naik, Anti-inflammatory activity of piperine, Jpn. J. Med. Sci. Biol. 43 (1990) 95–100.
[14] S.B. Jun, H.O. Da, M.C. Hyun, J.S. Bong, J.L. Sung, Y.K. Jung, I.Y. Hyung, C.Y. Myung, H.H. Dae, S.K. Kyoung, Anti-inflammatory and antiarthritic effects of piperine in human interleukin 1β-stimulated fibroblast-like synoviocytes and in rat arthritis models, Arthritis Res. Ther. 11 (2009) 49.
[15] X. Ying, K. Yu, X. Chen, H. Chen, J. Hong, S. Cheng, L. Peng, Piperine inhibited LPS induced expression of inflammatory mediators in RAW264.7 cells, Cell Immunol. 285 (2013) 49–54.
[16] Z. Wen-jun, Z. Zhen-biao, X. Nian-nian, G. Ying-fang, Q. Changwei, L. Cheng-ye, D. Gan-zhen, G. Meng-yao, Piperine plays anti-inflammatory role in *Staphylococcus aureus* endometritis by inhibiting activation an of NF-κB and MAPK pathways in mice, Evid. Based. Complement Alternat. Med. (2016) 10.
[17] S. Trifari, C.D. Kaplan, E.H. Tran, N.K. Crellin, H. Spits, Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells, Nat. Immunol. 10 (2009) 864–871.
[18] C. Gu-Trantien, S. Loi, S. Garaud, C. Equeter, M. Libin, A.D. Wind, CD4+ follicular helper T cell infiltration predicts breast cancer survival, J. Clin. Invest. 123 (2013) 2873–2892.
[19] P.S. Goedeqebuure, T.J. Eberlein, The role of CD4+ tumor-infiltrating lymphocytes in human solid tumors, Immunol. Res. 14 (1995) 119–131.
[20] W. Zheng, R.A. Flavell, The transcription factor GATA3 is necessary and sufficient for Th2 cytokine gene expression in CD4 T cells, Cell 89 (1997) 587–596.
[21] Z. Chen, A. Laurence, J.J. O'Shea, Signal transduction pathways and transcriptional regulation in the control of Th17 differentiation, Semin. Immunol. 19 (2007) 400–408.
[22] M. Haque, K. Fino, F. Lei, X. Xiong, J. Song, Utilizing regulatory T cells against rheumatoid arthritis, Front. Oncol. 4 (2014) 209.
[23] S. Eyerich, et al., Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling, J. Clin. Invest. 119 (2009) 3573–3585.
[24] A.M. Gilfillan, C. Tkaczyk, Integrated signalling pathways for mast-cell activation, Nat. Rev. Immunol. 6 (2006) 218–230.
[25] Y.A. Mekori, D.D. Metcalfe, Mast cells in innate immunity, Immunol. Rev. 173 (2000) 131–140.
[26] H. Nishikawa, S. Kitani, Tea catechins have dual effect on mast cell degranulation induced by compound 48/80, Int. Immunopharmacol. 8 (2008) 1207–1215.
[27] P. Mikolka, D. Mokra, A. Drgova, M. Petras, J. Mokry, Dimethyl sulfoxide in a 10% concentration has no effect on oxidation stress induced by ovalbumin-sensitization in a guinea-pig model of allergic asthma, J. Physiol. Pharmacol. 63 (2) (2012) 179–186.
[28] G.M. Bello, R.K. Winn, M. Jonas, E.Y. Chi, T.R. Martin, W.C. Liles, Fas (CD95) induces alveolar epithelial cell apoptosis *in vivo* - Implications for acute pulmonary inflammation, Am. J. Pathol. 158 (1) (2001) 153–161.
[29] Y.H. Choi, et al., Inhibitory effects of *Agaricus blazei* on mast cell-mediated anaphylaxis-like reaction, Biol. Pharm. Bull. 29 (2006) 1366–1371.
[30] H. Hachisuka, H. Nomura, F. Sakamoto, O. Mori, K. Okubo, Y. Sasai, Effect of antianaphylactic agents on substance P induced histamine release from rat peritoneal mast cells, Arch. Dermatol. Res. 280 (1988) 158–162.
[31] J. Harakal, C. Rival, H. Qiao, K.S. Tung, Regulatory T cells control Th2-dominant murine autoimmune gastritis, J. Immunol. 197 (1) (2016) 27–41.
[32] J.A. Woodfolk, Cytokines as a therapeutic target for allergic diseases: a complex picture, Curr. Pharm. Des. 12 (2006) 2349–2363.
[33] K. Oboki, T. Ohno, H. Saito, S. Nakae, Th17 and allergy, Allergol. Int. 57 (2) (2008) 121–134.
[34] H. Wakashin, et al., IL-23 and Th17 cells enhance Th2-cell-mediated eosinophilic airway inflammation in mice, Am. J. Respir. Crit. Care Med. 178 (2008) 1023–1032.
[35] A. Jamshidian, V. Shaygannejad, A. Pourazar, S.H. Zarkesh-Esfahani, M. Gharagozloo, Biased Treg/Th17 balance away from regulatory toward inflammatory phenotype in relapsed multiple sclerosis and its correlation with severity of symptoms, J. Neuroimmunol. 262 (2013) 106–112.
[36] J.A. Boyce, K.F. Austen, No audible wheezing: nuggets and conundrums from mouse asthma models, J. Exp. Med. 201 (2005) 1869–1873.
[37] A.B. Kay, The role of eosinophils in the pathogenesis of asthma, Trends Mol. Med. 11 (2005) 148–152.
[38] D.J. Shale, A.A. Lonescu, Mucus hypersecretion: a common symtom, a common mechanism? Eur. Respir. J. 23 (2004) 797–798.
[39] R. Pawankar, S. Mori, C. Ozu, S. Kimura, Overview on the pathomechanisms of allergic rhinitis, Asia Pac. Allergy 1 (3) (2011) 157–167.
[40] M.Y. Lee, I.S. Shin, H.S. Lim, H.K. Shin, A water extract of Samchulkunbi-tang attenuates airway inflammation by inhibiting iNOS and MMP-9 activities in an ovalbumin-induced murine asthma model, BMC Complement Altern. Med. 12 (2012) 257.
[41] C. Ma, Z. Ma, X.L. Liao, J. Liu, Q. Fu, S. Ma, Immunoregulatory effects of Glycyrrhizic acid exerts anti-asthmatic effects via modulation of Th1/Th2 cytokines and enhancement of CD4+ CD25+ Foxp3+ regulatory T cells in ovalbumin-sensitized mice, J. Ethnopharmacol. 148 (2013) 755–762.
[42] A.C. Roever, G. Heine, T. Zuberbier, M. Worm, Allergen-mediated modulation of CD23 expression is interferon-gamma and interleukin-10 dependent in allergic and non-allergic individuals, Clin. Exp. Allergy 33 (11) (2003) 1568–1575.
[43] P.C. Fulkerson, N. Zimmermann, E.B. Brandt, E.E. Muntel, M.P. Doepker, J.L. Kavanaugh, et al., Negative regulation of eosinophil recruitment to the lung by the chemokine monokine induced by IFN-γ (Mig, CXCL9), Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 1987–1992.
[44] O. Dienz, M. Rincon, The effects of IL-6 on CD4 T cell responses, Clin. Immunol. 130 (2009) 27–33.
[45] M.O. Li, R.A. Flavell, Contextual regulation of inflammation: a duet by transforming growth factor-beta and interleukin-10, Immunity 28 (4) (2008) 468–476.
[46] Y. Ogawa, E.A. Duru, B.T. Ameredes, Role of IL-10 in the resolution of airway inflammation, Curr. Mol. Med. 8 (5) (2008) 437–445.
[47] M. Pretolani, M. Goldman, Immunol. Today 18 (1997) 277–280.
[48] T. Kishimoto, IL-6: from its discovery to clinical applications, Int. Immunol 22

(2010) 347–352.
[49] M. Marini, E. Vittori, J. Hollemborg, S. Mottoli, Expression of the potent inflammatory cytokines, granulocyte/macrophage colony-stimulating factor and interleukin-6, and interleukin-8, in bronchial epithelial cells of patients with asthma, J. Allergy Clin. Immunol. 89 (5) (1992) 1001–1009.
[50] J.H. Wang, C.J. Trigg, J.L. Devalia, S. Jordan, R.J. Davies, Effect of inhaled beclomethasone dipropionate on expression of proinflammatory cytokines and activated eosinophils in the bronchial epithelium of patients with mild asthma, J. Allergy Clin. Immunol. 94 (1994) 1025–1034.
[51] W.J. Ming, L. Bersani, A. Mantovani, Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes, J. Immunol. 87 (1987) 1469–1474.
[52] V.J. Johnson, B. Yucesoy, M.I. Luster, Prevention of IL-1 signaling attenuates airway hyperresponsiveness and inflammation in a murine model of toluene diisocyanate-induced asthma, J. Allergy Clin. Immunol. 116 (2005) 851–858.
[53] U. Lappalainen, J.A. Whitsett, S.E. Wert, J.W. Tichelaar, K. Bry, Interleukin-1beta causes pulmonary inflammation, emphysema, and airway remodeling in the adult murine lung, Am. J. Respir. Cell Mol. Biol. 32 (2005) 311–318.
[54] L.J. Rosenwasser, Biologic activities of IL-1 and its role in human disease, J. Allergy Clin. Immunol. 102 (1998) 344–350.
[55] J.A. Schmidt, S.B. Mizel, D. Cohen, Interleukin-1, a potential regulator of fibroblast proliferation, J. Immunol. 128 (1982) 2177–2182.
[56] M.Y. Lee, J.A. Lee, C.S. Seo, H. Ha, N.H. Lee, H.K. Shin, Protective effects of Mentha haplocalyx ethanol extract (MH) in a mouse model of allergic asthma, Phytother. Res. 25 (2011) 863–869.
[57] N. Miyagawa, H. Iwasaki, T. Kato, M. Tanaka, T. Shibata, K. Wakitani, Induction of late airway response was involved in serum antigen-specific immunoglobulin G in rats, Int. Immunopharmacol. 8 (2008) 1848–1853.
[58] Y. Ishikawa, et al., Y. Nishimura, Antigen-Specific IgG ameliorates allergic airway inflammation via Fcg receptor IIB on dendritic cells, Respir. Res. 12 (2011) 42.
[59] S.J. Galli, M. Tsai, A.M. Piliponsky, The development of allergic inflammation, Nature 454 (2008) 445–454.