



Research paper

Piper Nigrum extract improves OVA-induced nasal epithelial barrier dysfunction via activating Nrf2/HO-1 signaling

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ABSTRACT

Background: *Piper nigrum* L. (Piperaceae) is commonly used as a spice and traditional medicine in many countries. It has been reported to have anti-oxidant, anti-bacterial, anti-tumor, anti-mutagenic, anti-diabetic, and anti-inflammatory properties. However, the protective role of *P. nigrum* on epithelial function of upper respiratory tract injury in an allergic rhinitis (AR) mouse model has been unclear. This study aims to investigate the effects of *P. nigrum* fruit extract (PNE) on the nasal epithelial barrier function of the upper respiratory tract in an ovalbumin (OVA)-induced AR model.

Methods: AR mouse model was established by intraperitoneal injection with 200 μ L saline containing 50 μ g OVA adsorbed to 1 mg aluminum hydroxide, and intranasal challenge with 20 μ L per nostril of 1 mg/ml OVA. Besides, mice were orally administrated once daily with PNE and dexamethasone (Dex) in 13 days. The nasal symptoms, inflammatory cells, OVA-specific immunoglobulins, cytokines, nasal histopathology, and immunohistochemistry were evaluated.

Results: The PNE oral administrations inhibited allergic responses via reduction of OVA-specific antibodies levels and mast cells histamine release, accordingly, the nasal symptoms in the early-phase reaction were also clearly ameliorated. In both nasal lavage fluid and nasal tissue, PNE suppressed the inflammatory cells accumulation, specifically with eosinophils. The intravenous Evans blue injection illustrated the epithelial permeability reduction of nasal mucosa layer in PNE-treated mice. Also; PNE treatments protected the epithelium integrity by preventing the epithelial shedding from nasal mucosa; as a result of enhancing the strong expression of the E-cadherin tight junction protein in cell-to-cell junctions, as well as inhibiting the degraded levels of zonula occludens-1 (ZO-1) and occludin into the nasal cavity. Additionally, PNE protected against nasal epithelial barrier dysfunction via enhancing the expression of Nrf2 activated form which led to increasing synthesis of the anti-inflammation enzyme HO-1.

Conclusions: These obtained results suggest that PNE has a promising strategy for epithelial barrier stabilization in allergic rhinitis treatment.

1. Introduction

P. nigrum L. known as black pepper, belongs to the Piperaceae

family that comprises over 1000 species with tropical and subtropical distribution. The coastal areas of India are its prime origin but nowadays its plantation is extended to other parts of the globe too like

Abbreviations: PNE, *Piper Nigrum* extract; Dex, dexamethasone; NALF, nasal lavage fluid; Nrf2, nuclear factor (erythroid-derived 2)-like 2; HO-1, heme oxygenase-1; ZO-1, zonula occludens-1; AR, Allergic rhinitis

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Vietnam, Ceylon, Malaysia, Indonesia, and Brazil [1]. Of the various spices, black pepper holds a prominent position and is acknowledged as “King of Spices” [2]. Actually, it is a very widely used spice and important healthy food owing to its antioxidant, antimicrobial, anti-carcinogenic, anti-inflammatory potential and gastro-protective modules [1,3]. Black pepper contains volatile oil, oleoresins, and alkaloids. Major alkaloids present in black pepper are piperine, chavicine, piperidine, and piperetine. The terpenes, steroids, lignans, flavones, and alkamides are other primary constituents. Nowadays, 46 compounds are discovered from the essential oil of black pepper [1]. Anti-inflammatory activities of black pepper are reported for the first time some 2 decades ago. Piperine from black pepper mitigates the acute inflammatory process, through stimulating the pituitary-adrenal axis [4]. Piperine at doses 20 and 100 mg/kg/day inhibited the interleukin, matrix metalloproteinase, prostaglandin E2, and activator protein 1 [5]. Recently, the previous data reported that piperine along with other components can prevent degenerative disorders like rheumatoid arthritis [6], suppressed the level of β -glucuronidase and lactate dehydrogenase in a dose-dependent manner [7], and showed an anti-inflammatory effect in *Staphylococcus aureus*-induced endometritis in mice models [8]. Overall, the anti-inflammatory properties of black pepper should be identified more clearly in specific diseases associated with inflammation such as allergic rhinitis.

Allergic rhinitis (AR) is a type I allergic disease of the nasal mucosa, characterized by paroxysmal repetitive sneezing, watery rhinorrhea, itching, and nasal blockage [9]. AR is a risk factor for asthma, it reduces the quality of life and school and work performance. The previous study reported that AR affects up to 40% of the world's population and over 500 million people suffer from AR worldwide and that the incidence is increasing [10,11].

In the early phase reaction of AR, antigens inhaled through the nasal mucosa pass through the nasal mucosal epithelial cells to bind to IgE antibodies on mast cells distributed over the nasal mucosa. In response to an antigen-antibody reaction, chemical mediators, such as histamine and peptide leukotrienes, are released from mast cells. These irritate the sensory nerve endings and blood vessels of the nasal mucosa to cause sneezing, watery rhinorrhea, and nasal mucosal swelling. In the late phase reaction, seen at 6–10 h after antigen exposure, various inflammatory cells, such as activated eosinophils, infiltrate into the nasal mucosa exposed to antigens in response to cytokines, chemical mediators, and chemokines [9].

There has been significant interest in the role the nasal epithelial barrier plays in airway diseases such as AR and asthma [12–14]. The barrier function of the airway epithelium depends on tight junction and adherens junction created by intercellular interactions of proteins such as zonula occludens-1, epithelial cadherin and occludin. These proteins function to limit intercellular passage of fluid, antigens, virus and protect the underlying tissue from exposure to noxious and allergenic stimuli [12]. The damage of tight junction is major cause of epithelial barrier breakdown during lung or nasal inflammation [13,14]. Collectively, airway epithelial barrier stabilization may improve AR pathophysiology.

Black pepper has been widely known spice and important healthy food owing to its antioxidant, antimicrobial, anti-carcinogenic, and anti-inflammatory potential [1,3], however, the anti-allergic rhinitis mechanism of *P. nigrum* has been currently unclearly. This present study aims to investigate the effects of *P. nigrum* extract (PNE) on the nasal epithelial barrier dysfunction of ovalbumin (OVA)-induced allergic rhinitis mouse model.

2. Materials and methods

2.1. Animals

Male six-week-old BALB/c mice were obtained from the Damool Science (Dae-jeon, Korea). The mice were maintained under standard

laboratory conditions for 1 week before experiments and were housed under an average temperature of 23 ± 3 °C, relative humidity of $50 \pm 10\%$ with 12 h light/dark cycles. All procedures of experiments were approved in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Chonbuk National University Medical School (CBN 2016-37) and were approved by the National Institutes of Health.

2.2. Preparation of PNE

Black pepper was purchased from a rural market (Gyeonggi-do, Korea). Raw PN material was extracted with $10 \pm 2 \times$ volumes of 70% ethanol under heating condition (65 ± 5 °C) for 5 ± 2 h, and the extract supernatants were collected using 5 μ m cartridge filter. This process was repeated. Next, the filtrate was concentrated by a rotary evaporator (IKA RV10, Staufen, Germany) and dried in a freeze dryer. The PNE powders were kept at 4 °C and managed with specimen number (KFRI-SL-1105) at the Korea Food Research Institute. For the AR mouse model treatment, the PNE powder was dissolved in saline to concentrations of 50, 100, and 200 mg/kg body weight. Dexamethasone (Dex) 2.5 mg/kg body weight was used as a positive control group that compared to PNE treatment groups.

2.3. Allergic rhinitis model establishment and treatment

BALB/c mice were divided into six groups (n = 9 or 10 each group): (1) Naive (2) OVA (3) PNE50 (4) PNE100 (5) PNE200 and (6) Dex. The OVA-induced AR mouse model and treatment were established as described in Fig. 1. Briefly, OVA-induced AR mice were sensitized by intraperitoneal injection with 200 μ L saline including 50 μ g OVA (Grade V, Sigma, St. Louis, MO, USA) adsorbed to 1 mg aluminum hydroxide (Thermo Scientific, Rockford, MD, USA) on day 1, 8, 15. Next, mice were orally administrated once daily with PNE (50, 100, and 200 mg/kg body weight) and Dex in 13 days, 1 h before the intranasal challenge of OVA. OVA group were given saline. One week after the last sensitization, on days 22 to 28, mice received an intranasal challenge with 1 mg/ml OVA, 20 μ L each nasal cavity. The 1% Evans blue solution was intravenously injected on day 28 for examining the nasal mucosal permeability. Also in order to confirm the protective effect of PNE on nasal epithelial barrier of OVA-induced allergic rhinitis via activating Nrf2/HO-1 signaling, ML385 (15 mg/kg body weight, MedChemExpress, NJ, USA), a special inhibitor of Nrf2, was

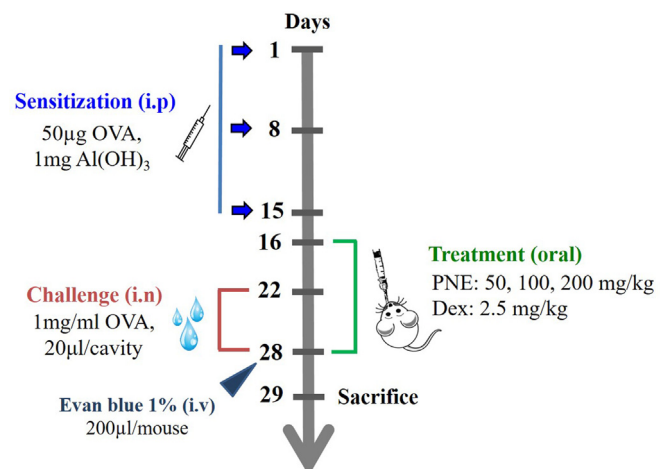


Fig. 1. Establishment of an allergic rhinitis mouse model and treatment with PNE. Mice were sensitized on days 1, 8 and 15, and challenged on days 22 to 28 with ovalbumin (OVA). Mice in the PNE or Dex groups were administered orally once daily at 50, 100, 200 mg/kg PNE or 2.5 mg/kg Dex for 13 days, respectively.

intraperitoneally injected 200 μ L on days 22, 24, 26, and 28, 30 min before PNE 200 mg/kg treatment. Finally, mice were sacrificed 24 h after the last OVA challenge. The frequency of nasal rubbing and sneezing were counted for 15 min on days 3, 5, 7 after OVA intranasal challenge.

2.4. Cell count for nasal lavage fluid

Immediately after sacrifice, nasal lavage fluid (NALF) was collected using an 18-gauge catheter. Tracheal was partially resected and inserted the catheter into the tracheal following the direction of the upper airway into the nasopharynx and perfused gently the nasal passages with 1 ml cold phosphate-buffered saline. Then, NALF was centrifuged at 10,000 RPM/minute for 10 min at 4 °C to collect the supernatant and stored at -70 °C for further studies. The cell pellets were resuspended in cold PBS for counting the total cell numbers using a hemocytometer. To examine differential cell number, 150 μ L of NALF was centrifuged onto slides using a cytospin device (Centrifuge 5403, Eppendorf, Hamburg, Germany) (1000 rpm, 10 min, 4 °C). Then the cell was stained by Diff-Quik Staining reagent (1-5-1 Wakinoama-Kaigandori, Chuo-Ku, Kobe, Japan) according to the protocols from the manufacturer. The inflammatory cells were counted at x400 magnification under a light microscope (Leica, USA).

2.5. Histological examination

For nasal histopathology examination, the heads were fixed in 10% neutral buffered formalin for 3 days at 23 $\pm$ 2 °C and decalcified in an ethylenediaminetetracetic acid decalcifying solution for 5 days at room temperature. The samples were dehydrated with a series of ethyl alcohol and xylene, then embedded in paraffin. The sections were cut at 5 μ m thickness. Then, the sections were stained with hematoxylin and eosin (H&E) (Sigma, St. Louis, MO) to assess general morphology, stained with Giemsa (Sigma, St. Louis, MO) to identify eosinophil accumulation and stained with periodic acid-Schiff (PAS) (Sigma, St. Louis, MO) for observation of goblet cell hyperplasia. The cells number were counted in the nasal septum and lateral process at 400 $\times$ magnification. For observation the Evans blue present in nasal mucosa, the sections were slightly stained with eosin for 5 s (Sigma, St. Louis, MO) to highlight the slight blue background of Evans blue dye.

2.6. Quantification of immunoglobulins, transcriptional factors and cytokines

The blood was harvested by cardiac puncture and was centrifuged to obtain the serum for immunoglobulins analysis. The supernatant was collected from the NALF for quantifying cytokines release. The levels of OVA-specific immunoglobulin (Ig) E, anti-OVA specific IgG1, anti-OVA specific IgG2a (R&D Systems Inc., Minneapolis, MN, USA) and mast cell histamine (Abcam Inc., Cambridge, MA, USA) were quantified using ELISA kits according to the manufacturers' instructions. Cytokine quantitation kits (BD Biosciences, San Diego, CA, USA) were used to measure transcription factor levels including Nrf-2, HO-1 and the tight junction-associated protein such as E-cadherin, ZO-1, occludin, according to manufacturer protocols. Briefly, supernatants and standard solutions were transferred to 96-well plates pre-coated with monoclonal antibodies to each of the target cytokines and incubated for 2 h at room temperature. After washing with the washing buffer included in the kit, a horseradish peroxidase-conjugated secondary antibody was added to each well, and incubation was continued at room temperature for 2 h. After removal of the secondary antibody and thorough wells washing, the enzymatic reaction was performed by adding the substrate solution, and samples were incubated for 30 min in the dark. The reaction was terminated by addition of the stop solution, and absorbance was measured at 450 nm in a microplate reader (Molecular Devices, Inc., Sunnyval, CA, USA).

2.7. Immunohistochemistry examination

For E-cadherin immunohistochemistry, the Rabbit specific HRP/DAB (ABC) Kit (Abcam Inc., Cambridge, MA, USA) and anti-E-cadherin antibody (Abcam Inc., Cambridge, MA, USA) were used. Briefly, the sections were heated by boiling water at 100 °C for 15 min in 1 $\times$ citrate buffer (Abcam Inc., Cambridge, MA, USA), pH 6.0, washed with PBS 2 times for 5 min each time, then blocked for 10 min in hydrogen peroxide, washed with PBS 3 times for 10 min each time. The samples were incubated protein block for 30 min at room temperature to block non-specific background staining. Next, the slides were incubated overnight at 4 °C in a humidified box with E-cadherin primary antibody (1:200), followed by rinsing with PBS 3 times for 5 min each time. The samples then were applied with biotinylated goat anti-polyvalent for 1 h 30 min at room temperature, washed with PBS 3 times for 5 min each time, and followed by streptavidin-peroxidase for 10 min. Thereafter, the samples were stained with 3,3'-diaminobenzidine, counterstained with hematoxylin, dehydrated in ethanol and xylene. Finally, the slides were mounted and observed under an optical microscope ($\times$ 400 magnification).

2.8. Nasal mucosa permeability analysis

To analyze nasal mucosa permeability, mice were sacrificed after 24 h of Evans blue dye injection, NALF was collected and quantified the its concentration using a spectrophotometer to detect the extravasation of Evans blue dye into the nasal cavity. The optical density was measured at a wavelength of 450 nm in a microplate reader (Molecular Devices, Inc., Sunnyval, CA, USA).

2.9. Western blotting analysis

For NRF2 protein detection, lung tissues were homogenized, and then their nuclear fractions were extracted by NE-PER Nuclear and Cytoplasmic extraction reagents (Thermo Fischer Scientific, Waltham, MA, USA). Total protein concentration from the lung tissues homogenate was determined by BCA assay according to the manufacturer's instructions. Protein levels were quantified using an automated capillary-base size sorting system (WES; ProteinSimple, Santa Clara, CA, USA). All procedures were performed according to the manufacturer's instruction. Four microliters of lung homogenate and primary antibodies [NRF2 (1:50) and Lamin B (1:50) (Cell signaling technology, Danvers, MA, USA)] were used to detect NRF2 protein. The data was analyzed with inbuilt Compass software (ProteinSimple).

2.10. RNA isolation from lung and quantitative real-time RT-PCR analyses

RNA from lung tissues was extracted and purified using the RNeasy Mini Kit (Qiagen, Germany) and cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Qiagen, Germany). RT-PCR of the sample was performed in a Rotor-Gene Qplex System (Qiagen, Germany) under the following thermal conditions; 10 min at 95 °C, then 45 cycles of 10 s at 95 °C and 15 s at 60 °C. Using assays on demand from Qiagen, the following genes were analyzed: β -actin as a house-keeping gene and HO-1. The sequences of the primers used were as follows: β -actin, F-5'-TGT CCA CCT TCC AGC AGA TGT-3' and R-5'-GCT CAG TAA CAG TCC GCC TAG-3'; HO-1, F-5'-AAG CCG AGA ATG CTG AGT TCA-3' and R-5'-GCC GTG TAG ATA TGG TAC AAG-3'. The level of β -actin was used to normalize the amounts of assayable RNA in each sample. The data are shown as the target gene's relative delta delta CT (ddCT) compared with that of the internal control. The quantity of gene in each sample was normalized to the quantity of β -actin. "Fold-induction" of each mRNA species was calculated as follows: d threshold cycle (dCt) = (Ct of target mRNA) - (Ct of β -actin); ddCt = (dCt of mRNA in target gene) - (dCt of mRNA in control gene); fold-induction = 2^{-ddCt} .

2.11. Statistical analysis

The results were analyzed using Graph Pad Prism software (v5.0, La Jolla, CA, USA). The statistical significance of the differences among groups was analyzed by one-way ANOVA, followed by Student's test. The data were presented as means $\pm$ standard error (SE) of independent experiments. Significance was considered at the 95% confidence level ($P < 0.05$).

3. Results

3.1. PNE reduced allergic nasal symptoms induced by OVA

To investigate the effect of PNE on the early-phase allergic symptoms, the rubbing and sneezing scores were counted for 15 min on times 3, 5, 7 days after OVA intranasal challenge. The results showed that OVA-induced AR mice significantly increased the frequencies of rubbing and sneezing on days 5 and 7 after OVA challenge compared to the Naive group. However, PNE administration dose-dependent manner was significantly reduced allergic nasal symptoms after 7 days challenge compared to OVA group. Similarly, the Dex treatment also displayed a significant inhibition effect on allergic symptoms in AR mice model (Fig. 2).

3.2. PNE inhibited the infiltration of differential inflammatory cells in NALF

To identify the effect of PNE oral administration on migration and accumulation of immune cells in the nasal cavity, the differential inflammatory cells and total cells number in NALF were quantified. In OVA group, the eosinophil (red arrows), total cells numbers, and NALF cell preparation had markedly increased compared to the Naive group. In contrast, PNE at doses of 50, 100, 200 mg/kg and Dex 2.5 mg/kg treatments significantly decreased these eosinophils in NALF when compared to OVA group (Fig. 3).

3.3. PNE suppressed the serum levels of antigen-specific-antibody and histamine

The levels of anti-OVA specific IgE, anti-OVA IgG1 and anti-OVA IgG2a in serum were examined to investigate the effect of PNE on allergic inflammatory responses. The data showed a significant up-regulation of anti-OVA IgE (Fig. 4A), anti-OVA IgG1 (Fig. 4B) and down-regulation of the anti-OVA-IgG2a level (Fig. 4C) in OVA group

compared with those in the Naive group. In contrast, PNE administrations at dose-dependently showed lower the levels of anti-OVA IgE, anti-OVA IgG1, and higher anti-OVA IgG2a in serum than those in the OVA group, similarly with Dex-treated mice. Also, the histamine release from mast cells was also significantly decreased in serum after PNE oral treatments (Fig. 4D). These results showed that PNE suppressed the allergic responses via modulating the serum immunoglobulin subset and mast cells histamine release.

3.4. PNE ameliorated the nasal mucosa thickness, the accumulation of eosinophils and the hyperplasia of goblet cells

H&E staining was performed to analysis the general morphological of the nasal cavity. Structure abnormalities were observed in the nasal mucosa of OVA group, the nasal septum region showed an increased accumulation of inflammatory cells in the subepithelium, led to a significant increase of mucosa thicknesses (double-headed arrow, Fig. 5A, D). Meanwhile, the nasal mucosa in PNE and Dex-treated mice were found thinner than those compared to OVA group (Fig. 5A, C). Giemsa staining revealed that oral administrations of PNE and Dex strongly suppressed eosinophilic infiltration into the nasal mucosa, while, in the AR mice, the presence of eosinophils was over-expression (Fig. 5B, E). Eosinophils were observed with red-staining cytoplasm and was marked with black head arrows (Fig. 5B). PAS staining showed a higher number of goblet cells in the nasal epithelium in AR group compare to those in the Naive and PNE treatment groups (Fig. 5F). The mucus hypersecretion which was observed with a violet color was highly expressive in the nasal mucosa epithelium of the OVA group, and it was ameliorated in PNE-treated mice (Fig. 5C). These results suggested that PNE administrations at dose-independent manner had the protective effect on nasal mucosa layer.

3.5. PNE ameliorated nasal mucosal permeability

Using intravenous Evans blue injection to evaluate the Evans blue diffusion onto nasal mucosa as well as Evans blue leakage into the nasal cavity. The histological analysis showed that OVA group was highly expressed of Evans blue in nasal mucosa, as a result of spreading Evans blue from blood vessels onto whole mucosa layer which was recognized by a slight blue background. In contrast, PNE treatment groups prevented the Evans blue extravasation from blood vessels to intercellular space, as a result of gradually decreasing blue color in epithelium layer (Fig. 6A). In addition, a significant increase of Evans blue concentration

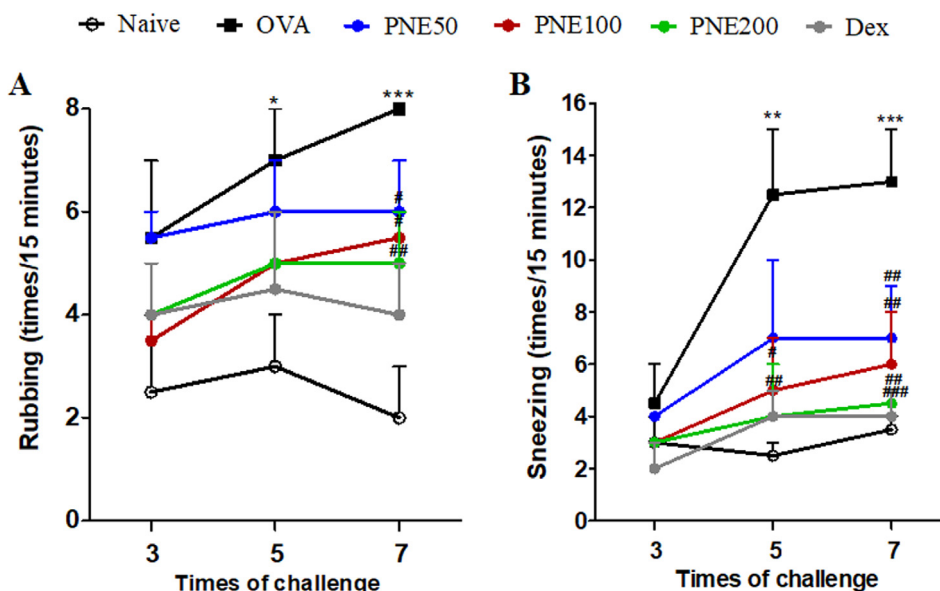


Fig. 2. PNE inhibited the OVA-induced allergic nasal symptoms. (A) Rubbing score. (B) Sneezing score. Rubbing and sneezing events were counted for 15 min after 3, 5, 7 time of OVA challenge. The values represent the mean $\pm$ SE ($n = 9$ or 10 /group). Significant differences at * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with the Naive group. # $P < 0.05$, ## $P < 0.01$, ### $P < 0.001$ compared with the OVA group.

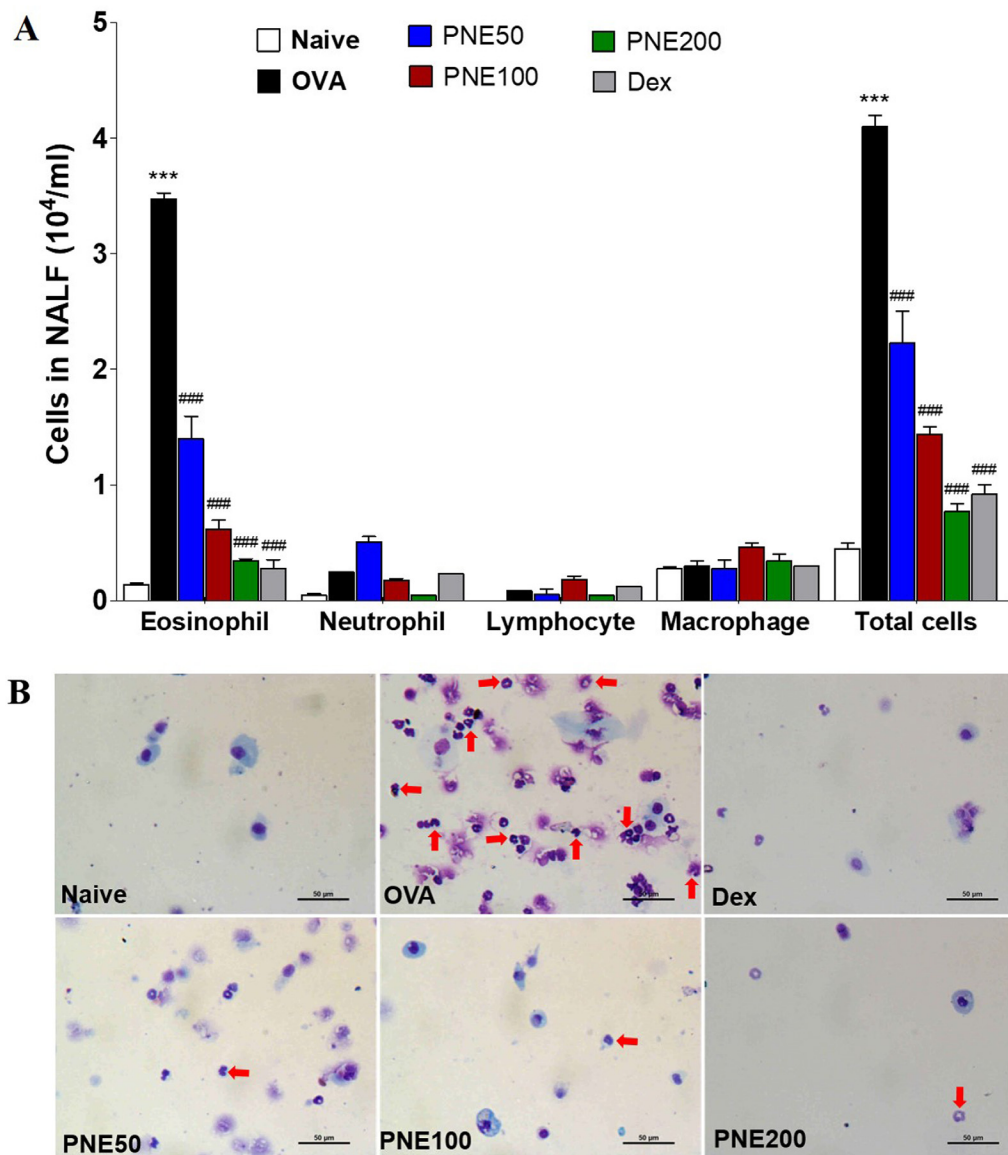


Fig. 3. PNE suppressed the infiltration of differential inflammatory cells and total cells in NALF (A). The differential cells were isolated using cytopsin and then stained with Diff-Quik (B). Red arrows indicate the eosinophils. The values represent the mean $\pm$ SE (n = 9 or 10/group). Significant differences at *** P < 0.001 compared with the Naive group. ### P < 0.001 compared with the OVA group. Scale bars: 50 μ m.

in NALF was observed in the OVA group. However, the dye concentration was significantly lower in PNE-treated mice compared to OVA group (Fig. 6B), indicating that PNE treatments can reduce the nasal mucosal permeability and prevent the Evans blue extravasation from epithelial layer into the nasal cavity.

3.6. PNE prevented nasal epithelial barrier dysfunction

The previous data revealed that Nrf2 was involved in regulating airway epithelial barrier integrity [14]. We assessed the Nrf2/HO-1 expression, nasal epithelium shedding, and tight junction proteins to know whether or not PNE could inhibit OVA-induced inflammatory mechanism. Exposure to OVA resulted in an increased exacerbation of nasal epithelium injury. Diff-Quik staining (Fig. 7A) and epithelium cell counting (Fig. 7C) revealed that a degree of the epithelial shedding from nasal epithelium layer was over-expressed in OVA group. These effects were reversed by PNE oral treatments, resulted in markedly inhibiting cells loss from epithelial layers, indicating that PNE protected nasal epithelial barrier integrity.

Besides, tight junction-associated proteins including E-cadherin, zonula occludens-1 (ZO-1) and occludin were found high-level expression in NALF of OVA-exposed mice, demonstrating that nasal mucosa was not maintained epithelium integrity and disrupted tight junctions between epithelial cell-cell contact sites. However, all these proteins were detected at low levels in PNE-treated mice at dose-independent manner when compared with OVA group (Fig. 7D–F).

Nrf2/HO-1 axis plays a crucial role in anti-inflammatory function. Our study showed that OVA-exposed mice were lower Nrf2 and HO-1 levels than Naive mice. In contrast, PNE-treated mice were significantly elevated the Nrf2 and HO-1 levels compared to OVA group (Fig. 7G, H).

Taken together, the data suggested that PNE oral administration is effective in protecting the barrier function of nasal mucosa through activating Nrf2/HO-1 signaling.

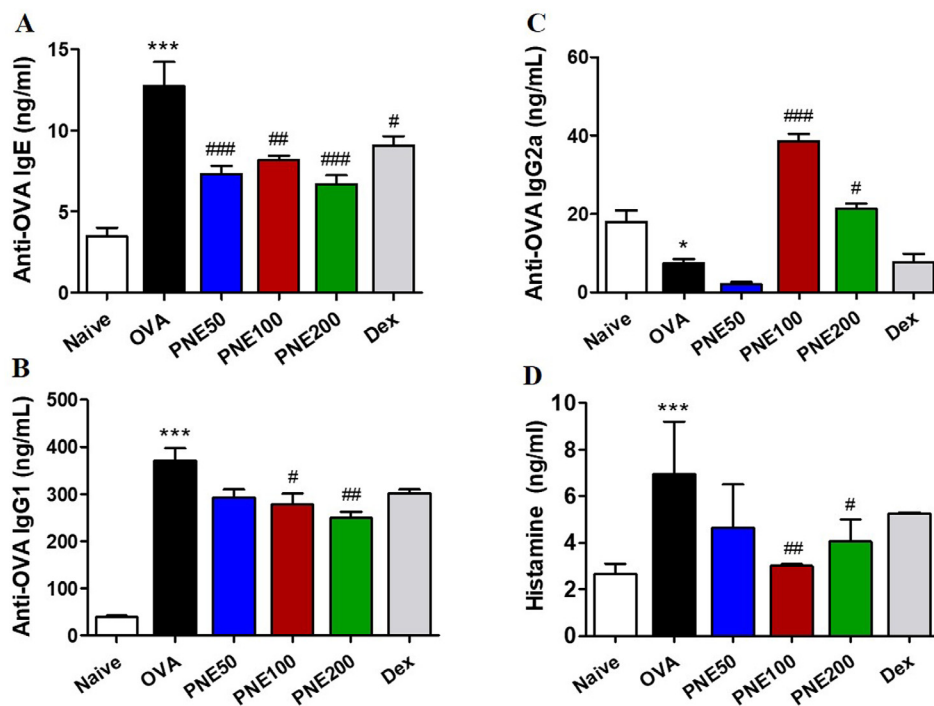


Fig. 4. PNE suppressed the allergic responses via regulating the levels of OVA-specific antibodies and mast cell histamine release. The levels of anti-OVA specific IgE (A), IgG1 (B) and histamine in serum (D) were decreased, and the levels of anti-OVA specific IgG2a (C) was increased in PNE-treated mice. The values represent the mean $\pm$ SE (n = 9 or 10/group). Significant differences at * P < 0.05, *** P < 0.001 compared with the Naive group. # P < 0.05, ## P < 0.01, ### P < 0.001 compared with the OVA group.

3.7. PNE attenuation of nasal epithelial barrier dysfunction by OVA in allergic rhinitis mouse is associated with the activation of Nrf2/HO-1 signaling

Currently, the inhibitory effect of PNE on nasal epithelial barrier disruption via modulating Nrf2/HO-1 signaling is still especially lacking *in vivo* direct evidence. In order to further explore this issue, an Nrf2 inhibitor, ML385, was intraperitoneally injected into OVA-sensitized and PNE-treated mice in order to inhibit Nrf2/HO-1 signaling. As shown Fig. 8, ML385 treatment increased the total cells of NALF (Fig. 8A and B), the serum levels of OVA-specific IgE/IgG1 (Fig. 8C–E), the degraded levels of tight junctions proteins (Fig. 8F–H) compared with only PNE group. Although ML385 treatment did not affect the histamine level (Fig. 8I), ML385 treatment decreased the levels of Nrf2 and HO-1 (Fig. 8J and K), a downstream of Nrf2 signaling, compared with PNE group. Also, in order to support the hypothesis that Nrf2 activity is involved in the antiinflammatory properties of PNE, we confirmed the nuclear translocation of Nrf2 and mRNA HO-1 expression driven by Nrf2 activation. Nrf2 translocation was decreased in OVA group, meanwhile the administration of PNE promoted the translocation of Nrf2. However, ML385 inhibited the translocation of Nrf2 promoted by PNE (Fig. 8L). In addition, mRNA expression level of HO-1 driven by Nrf2 activation was decreased in OVA group, meanwhile the administration of PNE increased the HO-1 mRNA expression. Nevertheless ML385 inhibited the translocation of Nrf2 promoted by PNE (Fig. 8M). Taken together, ML385 reversed PNE-regulated activity of Nrf2/HO-1 signaling as well as decreased the therapeutic effect of PNE on OVA-induced AR. These results suggested that Nrf2/HO-1 activation might be the underlying mechanism of PNE in the amelioration of OVA-induced AR.

4. Discussion

In this study, we investigate the antiallergic, anti-inflammatory functions of PNE using an OVA-induced AR mouse model. To demonstrate the effect of PNE on nasal epithelial barrier dysfunction of OVA-induced allergic responses, we evaluated nasal symptoms, OVA-specific antibodies, infiltration of inflammatory cells into the nasal mucosa, and epithelial barrier integrity. Also, the anti-inflammatory effect was

detected by assessing the Nrf2/HO-1 signaling pathway.

An allergic response is often divided into two phases including early-phase reaction and late-phase reaction. The early-phase reaction occurs within minutes of exposure to the allergen. In the early-phase of AR, mast cell degranulation following IgE-mediated cross-linking of the membrane-bound IgE high-affinity receptor (FcεRI) release granules stored mediators in mast cells within minutes, then histamine secretion induces early-phase symptoms such as sneezing, itching and runny nose [15–17].

In our study, mice were challenged intranasally with OVA for 7 days, we found an elevation of serum OVA-specific IgE, OVA-specific IgG1 levels and histamine release in OVA group, but not in PNE-treated mice. Furthermore, the down-regulation of serum OVA-specific antibodies and histamine levels were consistent with the reduction of allergic symptoms including sneezing and rubbing scores in PNE groups. These results indicated that PNE oral administrations could suppress the allergic responses in the early-phase of AR.

Late-phase reaction typically develops within 2 to 9 h after allergen exposure and was characterized by an accumulation of eosinophils in the nasal cavity. Eosinophils-derived mediators induce epithelial damage, resulting in nasal mucosal swelling [15,16,18]. The previous studies had shown that the proportions of eosinophils and neutrophils were higher in asthmatic airways with high concentrations of mast cells histamine [19]. In this study, the eosinophils number were accessed in the observed count of the histological section and in NALF. OVA-exposed mice showed an increased accumulation of eosinophils in both NALF and nasal mucosa layers, according to these results, the nasal mucosa layers were be swelling with high infiltration of inflammatory cells. Meanwhile, PNE-treated mice at dose-independent manner were effective in suppressing nasal eosinophilia and nasal thickness. Also, the decrease of nasal eosinophils was correlated with the serum histamine reduction which took place in the early-phase after treatment. According to these results, suggesting that PNE oral treatments could inhibit the allergic responses in both early-phase and late-phase of AR.

Recently, several studies showed that a defective epithelial barrier resulted from tight junction and/or adherence junction defects which are part of the underlying pathologic mechanism in airway disease like asthma and AR [20,21]. The early-phase of AR is initiated by an IgE-mediated mast cell degranulation with histamine secretion and is

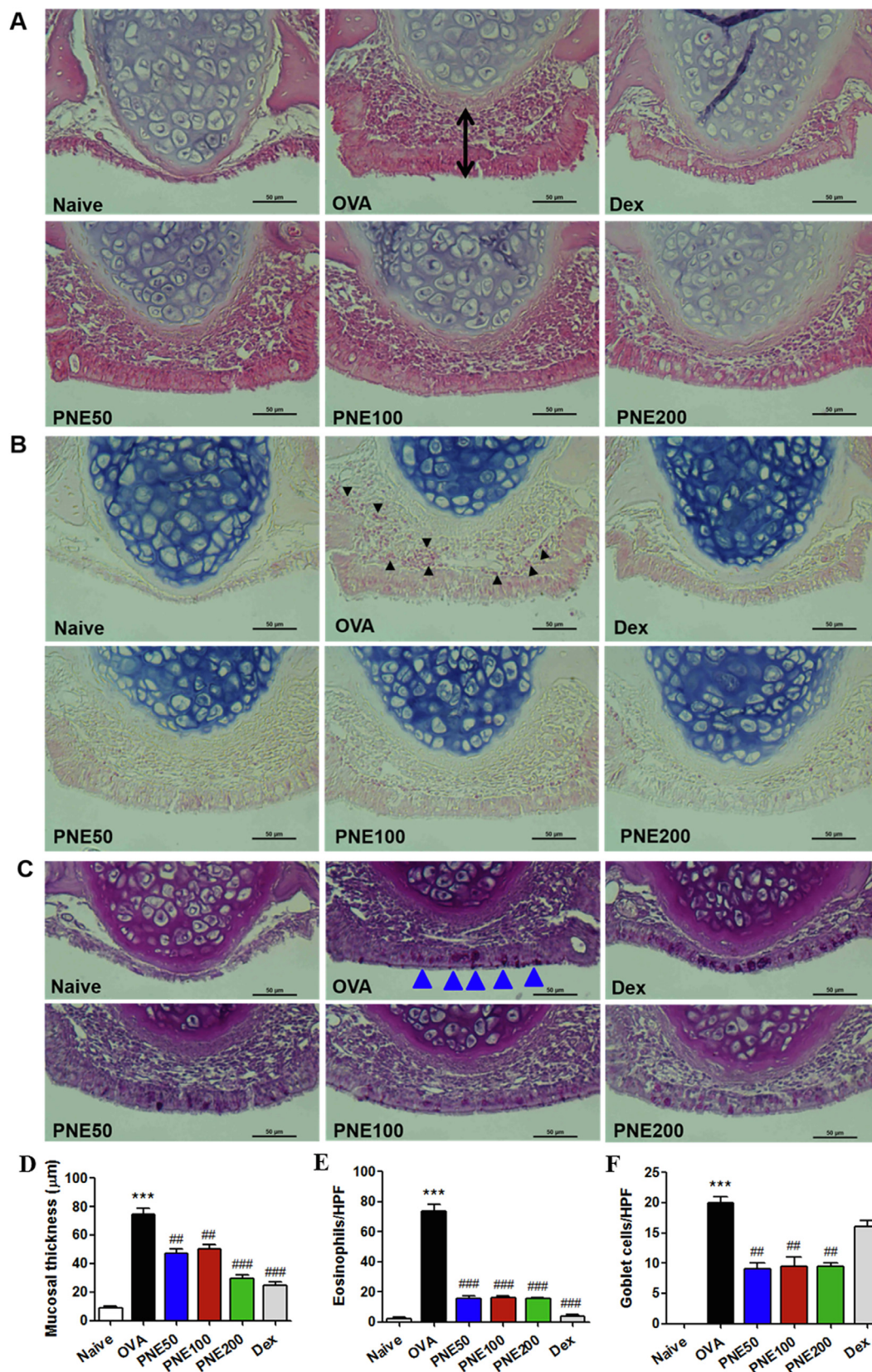


Fig. 5. PNE ameliorated the nasal mucosa swelling and accumulation of eosinophils and goblet cells. (A) Nasal mucosa was observed by H&E staining. Mucosa thickness of the septal region was measured higher in OVA group (double-headed arrow) and was ameliorated in PNE-treated mice at dose-independent manner. (B) Eosinophils were identified by Giemsa staining. The arrow-heads marked the eosinophils which were observed as red color. Eosinophils number was a high expression in the OVA group and ameliorated after PNE treatments. (C) Goblet cells were identified by PAS staining. The blue arrow-heads marked the mucus, observed as violet color. Scale bars: 50 μm. (D) Nasal mucosal thickness. (E) Eosinophils number in the nasal mucosa. (F) Goblet cells number in the nasal mucosa. The values represent the mean $\pm$ SE (n = 9 or 10/group). Significant differences at *** P < 0.001 compared with the Naive group. ## P < 0.001, ### P < 0.01 compared with the OVA group.

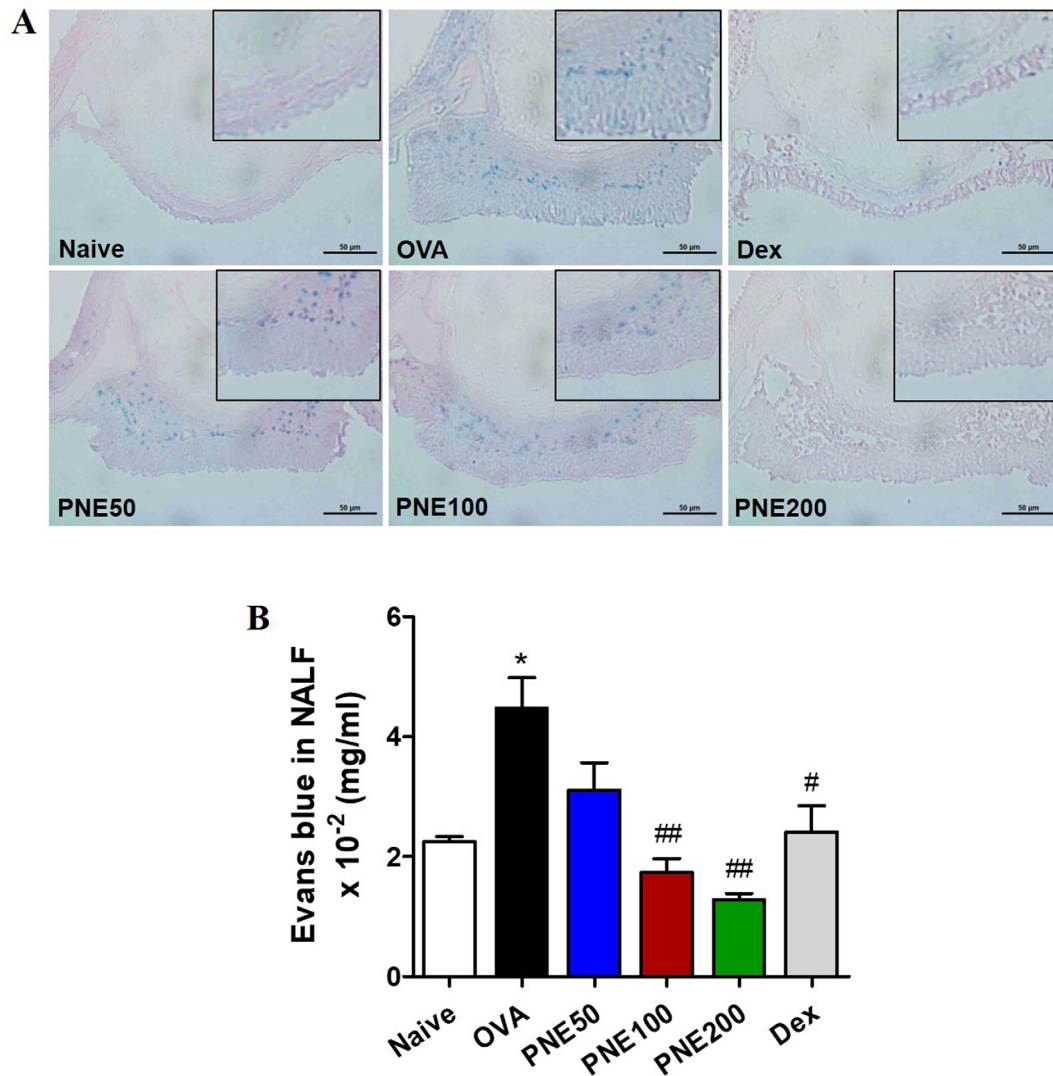


Fig. 6. PNE ameliorated nasal mucosal permeability. (A) The histological analysis showed the diffusion of Evans blue onto nasal mucosa tissue. PNE gradually decreased the blue background color in the epithelium layer by preventing the Evans blue extravasation from blood vessels onto intercellular space. (B) The Evans blue concentration in NALF. PNE-treated mice were significantly lower Evans blue concentration than compared to OVA group. Scale bars: 50 µm. The values represent the mean $\pm$ SE (n = 9 or 10/group). Significant differences at * P < 0.05 compared with the Naive group. # P < 0.01, ## P < 0.05 compared with the OVA group.

further progressed by Th2 and Th17 cytokines such as IL-4, IL-13, and IL-17 [15–17,20]. These cytokines maintain and aggravate a inflammatory condition in the nasal mucosa by inducing the infiltration of inflammatory cells and the disruption of an intact mucosal barrier [21,22]. Antagonizing IL-4 and IL-13 in vitro and anti-IL-4 treatment in mice prevented epithelial barrier disruption and hence represents a possible strategic target for breaking persistent nasal inflammation in AR [22].

Furthermore, the recent observation that Nrf2/HO-1 signaling plays a major role in the anti-inflammatory function and involves in regulating airway epithelial barrier integrity [14]. House dust mite-induced sinonasal epithelial cell barrier dysfunction may be preventable by Nrf2 activation and then Nrf2 antioxidant pathway may be a potential therapeutic target for nasal inflammation [23]. The elevation of HO-1 expression which is mediated by activated Nrf2 resulted in the reduction of tight-junction dysfunction in rat liver transplantation model [24]. Our previous studies reported that PNE prevented the activation of STAT3 and NF- κ B signaling which led to increasing Th1 cytokines and suppressing Th2 cytokines of AR mice [25] and ameliorated allergic inflammation through inhibiting the Th2/Th17 responses

and the mast cell activation of asthma mice [26]. Therefore, we hypothesized that PNE may have a protective effect on OVA-induced nasal epithelial barrier dysfunction via modulating Nrf2/HO-1 signaling. Indeed, PNE treatment increased the expression of Nrf2 and HO-1 that had been decreased by OVA in the present study. Besides, epithelial tight junctions are formed between adjacent cells by the interactions of a variety of transmembrane proteins, most commonly occludin, zonula occludens and cadherin [27,28]. The previous report has found that Nrf2 knockdown cell had suppressed the accumulation of tight junction and adherent junction proteins such as E-cadherin and ZO-1, that let to reduce the formation and enhancement of airway epithelial barrier integrity [14]. It showed that transcription factor Nrf2 plays important role in the regulation of airway epithelial barrier function. And the up-regulation of Nrf2 may lead to enhance the maintenance of cell-cell junctions in nasal epithelial barrier layers. In our study, PNE enhances the protective role of Nrf2 via decrease exacerbation of the epithelial shedding from nasal mucosa. As the result of limiting epithelial loss, the levels of tight junction-associated protein including E-cadherin, ZO-1, and occludin were also significantly decreased in PNE-treated mice. The immunostaining clarified more about PNE role on protecting E-

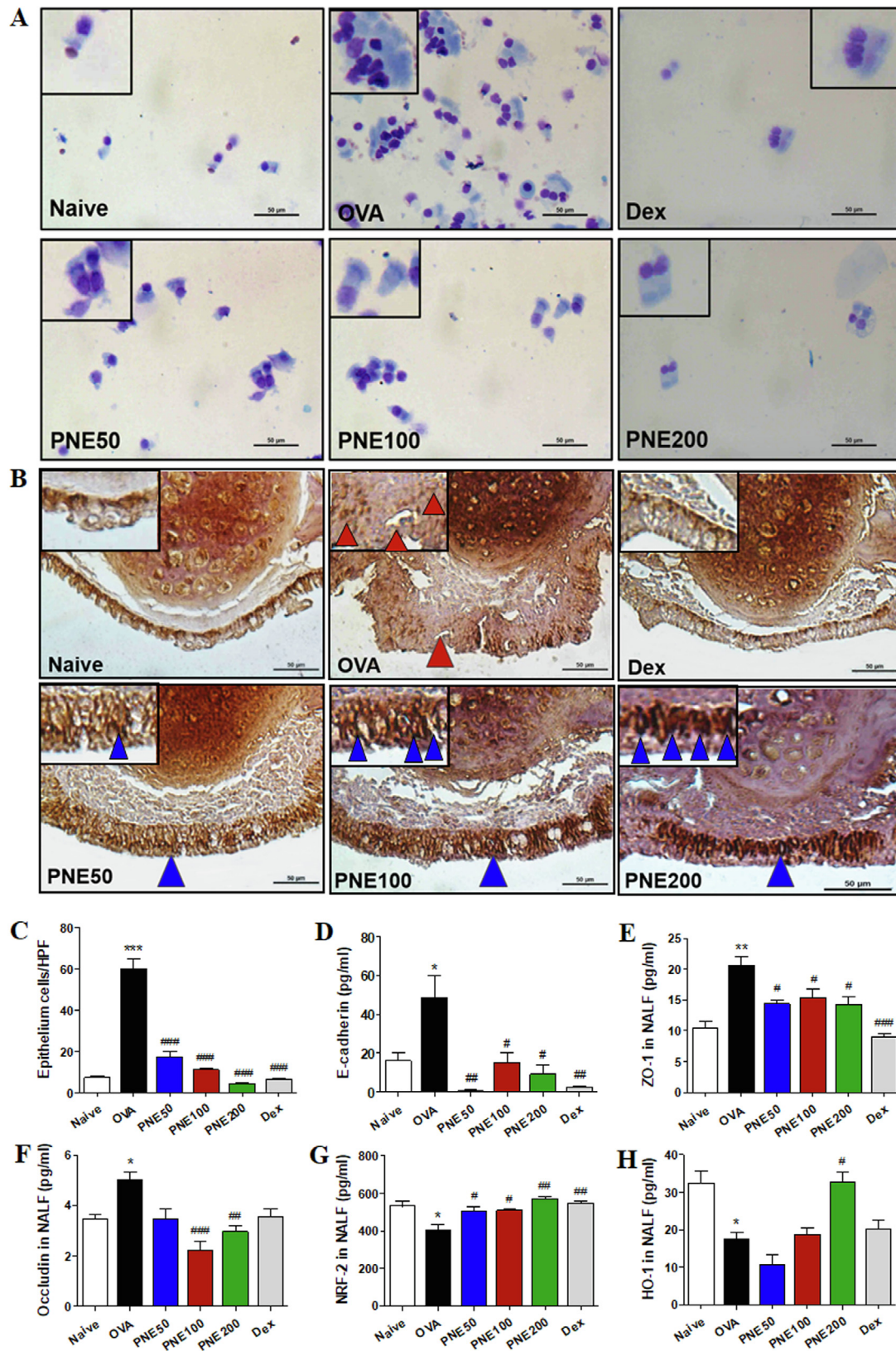


Fig. 7. PNE enhanced the activation of Nrf2/HO-1 signaling and prevented the nasal epithelial barrier dysfunction. The epithelial cells in NALF (A, C). The epithelial shedding was over-expressed in the OVA group and ameliorated in PNE-treated mice. The immunohistochemistry staining showed E-cadherin expression in epithelium layer (B). The red arrowhead indicates the disruption of E-cadherin in the OVA group. The blue arrow-head indicate the integrity of E-cadherin that can be observed as dark brown color in PNE-treated mice. Degraded tight junction-associated proteins including the E-cadherin, ZO-1, and occludin, respectively (D, E, F). Tight junction proteins were high-level expressions in NALF of OVA-exposed mice and clearly decreased in PNE treatments, indicating that PNE could prevent epithelium disruption in the nasal mucosa. The level of Nrf2 active forms in NALF was increased in PNE treatments (G). HO-1 protein in NALF was elevated in PNE treatment 200 mg/kg concentration (H). Scale bars: 50 μ m. The values represent the mean $\pm$ SE (n = 9 or 10/group). Significant differences at * P < 0.05, ** P < 0.01, *** P < 0.001 compared with the Naive group. # P < 0.05, ## P < 0.01, ### P < 0.001 compared with the OVA group.

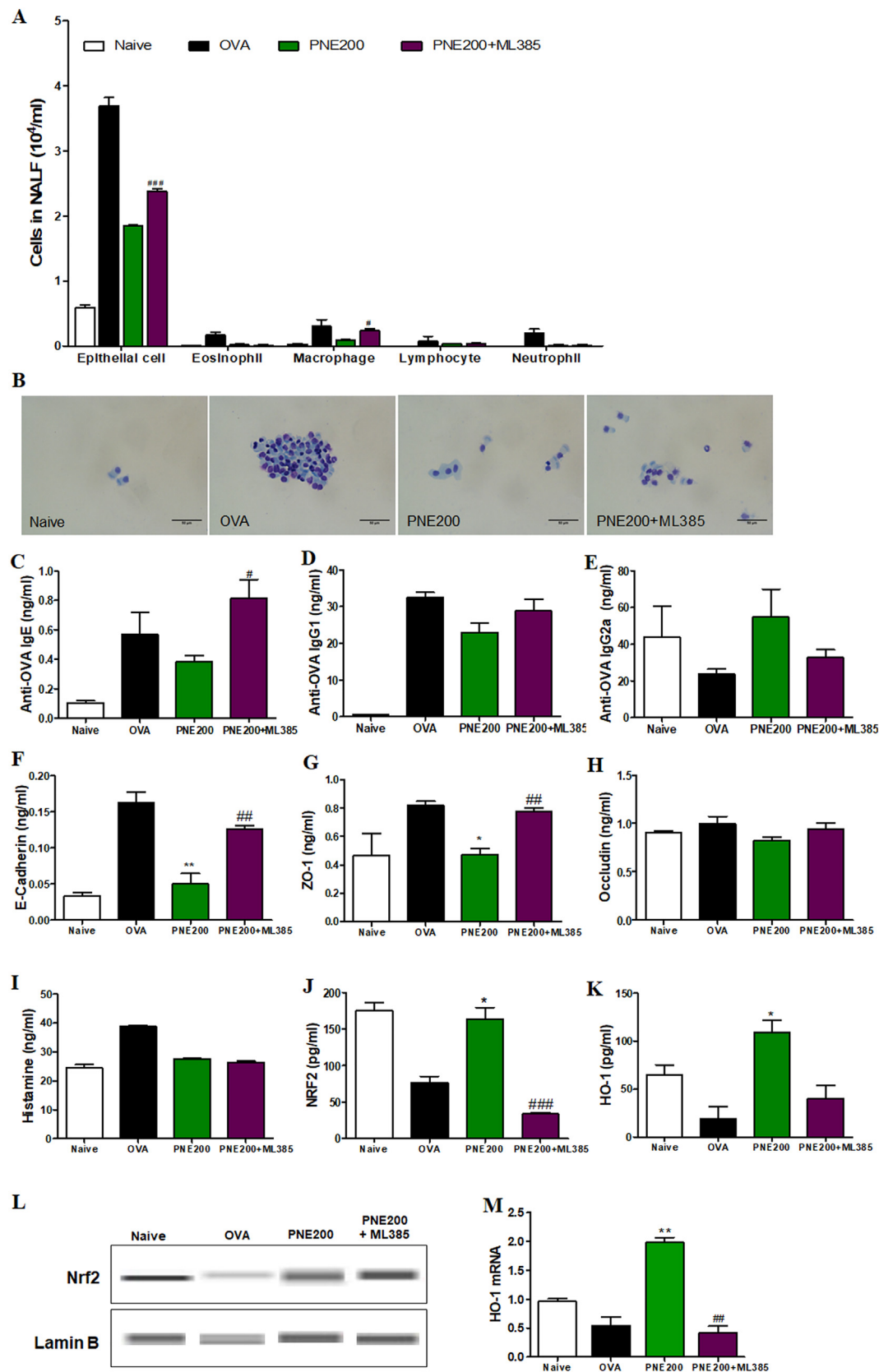


Fig. 8. Nrf2 inhibitor ameliorated the inhibitory effect of PNE on the OVA-induced nasal inflammation. Differential inflammatory cells in NALF (A). Diff-Quick-stained cytopsin (B). Anti-OVA IgE in serum (C). Anti-OVA IgG1 in serum (D). Anti-OVA IgG2a in serum (E). E-Cadherin in NALF (F). ZO-1 in NALF (G). Occludin in NALF (H). Histamine in serum (I). NRF2 in NALF (J). HO-1 in NALF (K). Nuclear Nrf2 in lung homogenate (L). HO-1 mRNA in lung homogenate (M). The Values represent the mean $\pm$ SE (n = 5/group). Significant differences at * P < 0.05, ** P < 0.01 compared with the OVA group. # P < 0.05, ## P < 0.01, ### P < 0.001 compared with the PNE200 group.

cadherin integrity status in epithelial cells. On the other hand, the extravasation of Evans blue onto a nasal epithelial tissue and nasal cavity were evaluated to supplement credibility for the protective role of PNE on epithelium barrier function. In OVA group; we found an increase the presence of Evans blue in nasal mucosa tissue which passed through the intercellular route, as a result of reducing junctions between mucosal cells to one adjacent another; as well as an increase of Evans blue concentration in nasal cavity caused by the breakdown in epithelial barrier function. The PNE treatments inhibited the Evans blue leakages from blood vessels to nasal mucosa tissue and from epithelial cell layer to the nasal cavity. These results reinforced the protective effect of PNE treatment on the epithelial barrier. Thus, PNE promoted the activation of Nrf2/HO-1 in this study might be explained the reduced nasal epithelial permeability and the inhibited epithelial barrier disruption, the suppressed activities of NF- κ B and Th2/Th17 responses. To further make sure our inference, ML385, a special inhibitor of Nrf2, was used in our experiment. The results showed that ML385 significantly suppressed the activations of Nrf2 and HO-1 signaling which activated by PNE, and also considerably increased the nasal epithelial shedding and the disruptions of tight junction proteins including ZO-1 and E-cadherin which suppressed by PNE. These data suggested that ML385 markedly reversed the effect of PNE on the Nrf2/HO-1 signaling, and the reduction of Nrf2 activity could increase tight junction disruption, nasal epithelial permeability, and epithelial barrier dysfunction. In addition, ML385 reversed PNE-regulated activity of Nrf2/HO-1 signaling as well as decreased the therapeutic effect of PNE on OVA-induced AR.

These results suggested that PNE could inhibit OVA-induced epithelial barrier dysfunction by regulating Nrf2/HO-1 signaling activation.

5. Conclusion

Overall, *Piper Nigrum* fruit extract has a positive effect on regulating the allergic responses by suppressing the OVA-specific antibodies, serum histamine release, and inflammatory cells accumulation. Additionally, *Piper Nigrum* fruit extract protected against epithelial barrier dysfunction, as a result of nasal histopathological amelioration via enhancing the activation of Nrf2/HO-1 signaling. These obtained results suggested that PNE may provide a promising strategy for immunotherapy in airway diseases such as allergic rhinitis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cellimm.2019.104035>.

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