Original Article
Apigenin attenuates PM2.5-induced airway hyperresponsiveness and inflammation by down-regulating NF-κB in murine model of asthma

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Abstract: The purpose of this study was to investigate the anti-inflammatory potential of the natural flavonoid apigenin to mitigate the airway inflammation in asthmatic mice exposed to particulate matter (PM) 2.5, and examine the possible mechanisms involved. BALB/c mice were sensitized and challenged with ovalbumin (OVA), then administered apigenin at a dose of 20 mg/kg/day, followed by PM2.5 exposure at a dose of 100 µg/mouse before prior to each challenge. The results showed that PM2.5 exposure aggravated airway hyper-responsiveness (AHR) and led to a mixed T helper (Th)2 cell/interleukin (IL)-17 response in asthmatic mice. Apigenin treatment markedly decreased both AHR and the percentage of eosinophils, as well as neutrophil infiltration in the bronchoalveolar lavage fluid (BALF) and lung tissue of OVA-sensitized and PM2.5-exposed mice. There were significant reductions in the levels of total serum immunoglobulin IgE and T-helper cell type 2 (Th2)-related cytokines (IL-4, IL-13) and Th17-related cytokine IL-17 in BALF. In addition, treatment with apigenin down-regulated the expression of nuclear factor kappa-light -chain-enhancer of activated B cells (NF-κB) p65 submit in lung tissue of asthmatic mice. These data suggest that apigenin exhibited both anti-allergic and anti-neutrophil-related inflammatory activity in a murine asthma model exposed to PM2.5, possibly through modulating IL-17 and down-regulating the expression of NF-κB. Thus, apigenin may be a promising candidate for preventing PM2.5 exposure-enhanced pre-existing asthma.

Keywords: Asthma, particulate matter 2.5, apigenin, airway hyperresponsiveness, mixed airway inflammation

Introduction

Asthma is a common pulmonary inflammatory disease, the prevalence of which has risen markedly worldwide; it was estimated in 2017 that ~235 million individuals had asthma [1]. The primary feature of asthma is chronic airway inflammation. Infiltration of eosinophils, mast cells, and T lymphocytes typically underlies the pathogenesis of allergic airway inflammation [2], which is associated with increased T helper (Th)2 cell cytokines [3]. Interleukin (IL)-4 and IL-13 are Th2-related cytokines that are known to be vital in eosinophil accumulation and are key factors involved in immunoglobulin (Ig)E synthesis. They may also contribute to airway hyper-responsiveness (AHR), mucus production and bronchial fibrosis [4]. Since asthma is

established as a heterogeneous disease, besides inflammation of eosinophils, neutrophilic airway inflammation serves a crucial role in certain stages underlying severe and acute exacerbations of asthma [5], which is responsible for poor response to glucocorticoid treatment. When secreted by a distinct cluster of differentiation (CD)4+ Th cell subset of Th17 cells, IL-17 (also known as IL-17A) contributes to neutrophilic asthma severity and enhances the migration and recruitment of neutrophils [6]. Previous studies have suggested that the level of IL-17 was associated with asthma severity and neutrophilic infiltration [7, 8]. Importantly, asthmatics with a mixed T helper (Th)2 cell/interleukin (IL)-17 response proved to have lower lung function and the worst asthma control. Nuclear factor κ-light chain-enhancer of activated B cells
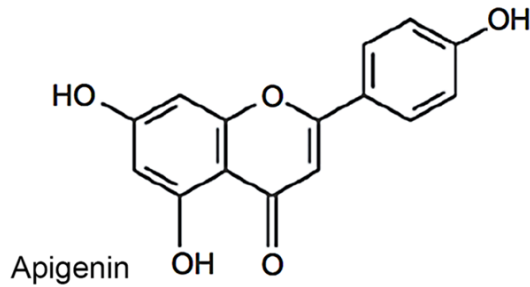
Figure 1. Chemical structure of apigenin.

(NF-κB) serves a key role in regulating the transcriptional process of NF-κB-dependent genes that encode proteins such as IL-4, IL-13, and tumor necrosis factor (TNF)-α [9, 10]. Thus, treatment strategies aimed at inhibiting Th2/IL-17 response and NF-κB activity would be effective [11].

Due to rapid industrialization, urbanization, and population growth, increased atmospheric pollution has been suggested to be the major risk factor for the development of allergic diseases. Particulate matter (PM) is a major component of air pollution. Based on its aerodynamic diameter, PM is categorized as coarse PM (aerodynamic diameter of 2.5-10 μm), fine PM (aerodynamic diameter of < 2.5 μm) and ultrafine PM (aerodynamic diameter of < 1 μm). The toxicity of PM is strongly associated with its size and components, and PM2.5 is small enough to penetrate terminal bronchioles and alveoli. The components of PM2.5 may include metals, sulfate, and organic carbon. Epidemiological studies have reported that PM2.5 can exacerbate asthma [12, 13]. In an *in vivo* study, PM2.5 was reported to exacerbate airway allergic inflammation, characterized by increased levels of inflammatory cells and cytokines [14]. However, few studies have focused on the effect of PM2.5 on mixed eosinophil and neutrophilic airway inflammatory phenotype in an asthmatic murine model, and the mechanism associated with PM2.5-induced inflammation is not well understood. Therefore, a purpose of the present study was to investigate all aspects of the PM2.5-induced exacerbation of asthma in a mouse model, and the role of NF-κB in this exacerbation.

Flavonoids comprise a large group of secondary plant metabolites and have been reported to have antioxidant, anti-bacterial, anti-viral and anti-inflammatory properties [15, 16]. Api-genin (**Figure 1**), a nonmutagenic and low toxicity flavone, is present in a variety of fruits and vegetables, including onions, parsley and oranges, chamomile tea, wheat sprouts, and certain seasonings. A previous study by our group demonstrated that apigenin had anti-inflammatory activity in a murine asthma model, and switched the immune response to allergens toward a Th1 profile [17]. Additionally, apigenin has been reported to inhibit the transforming growth factor β1-induced expansion of hyper-contractile α-smooth muscle actin and the proliferation of airway smooth muscle cells *in vitro* [19, 20]. While apigenin appears to possess multi-targeted activity, whether or not it may be benefit in the mediation of inflammatory responses induced by PM2.5 exposure remains unclear. In the present study, the anti-inflammatory potential of apigenin in preventing PM2.5 exposure-enhanced AHR and the corresponding inflammatory response was examined in asthmatic mice.

Materials and methods

Preparation of PM2.5 and measurement of components

PM2.5 was collected with a high-volume air sampler (Laoying 2033B, Qingdao, China) from January 1st to December 21st, 2016 in Yantai, China. The PM2.5 was gathered at a flow rate of 100 l/min for a continuous 8 h period each day. For extraction, the fiber filters were cut into pieces and immersed in Millipore water. Particles were extracted from the water by sonication using ultrasound frequencies for 1 h on ice. The water containing extracted PM2.5 was centrifuged at 5,000× g at 4°C, and then concentrated by freeze-drying [21]. Metals in the samples were determined using inductively coupled plasma atomic emission spectrometry (61E Trace and ICP-750, Termo Jarrell-Ash, Franklin, MA, USA). The concentrations of polycyclic aromatic hydrocarbons (PAHs) in the samples were analyzed using high-performance liquid chromatography (Hitachi Model 600 HPLC, Hitachi, Japan). The freeze-dried particulate matter was mixed with saline to obtain a particle suspension for subsequent experiments.

Experimental animals

A total of 60 female BALB/c mice (6-weeks-old, 18-22 g) were purchased from Shanghai La-
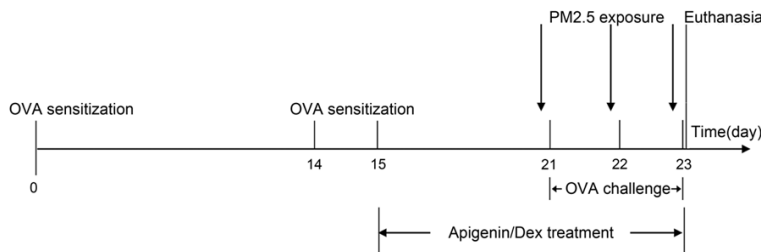
Figure 2. Overview of experimental design.

boratory Animals Center (Shanghai, China) and housed in plastic cages. The animal experimental protocol was approved by Nanjing Medical University (Nanjing, China) according to the guidelines of the Institutional Animal Care and Use Committee of Nanjing Medical University (permit no: 20110217). Animal experiments were performed in accordance with the regulations in the Guidance Suggestions for the Care and Use of Laboratory Animals of the People's Republic of China. The study protocols were performed under anesthesia when needed and conducted in such a way as to minimize suffering. The mice were kept under a 12-h light/dark cycle at a constant temperature of $22\pm2°C$ and relative humidity of $55\pm10\%$. All animals were provided free access to food and water.

Allergen sensitization/challenge, PM2.5 exposure, and apigenin treatment

Based on a previous OVA-induced mouse model of asthma [17], we made minor changes to establish the asthmatic model of exposure to PM2.5 [18]. The mice were acclimatized for 5 days prior to the initiation of the study, then were randomly divided into the following five groups (n=12/group): A control group, an OVA group (OVA-sensitized and challenged only), a PM2.5 group (OVA and PM2.5 co-exposure), an apigenin group (OVA, PM2.5 co-exposure and apigenin treatment), and a dexamethasone group (OVA, PM2.5 co-exposure and dexamethasone treatment). Mice in the last four groups were sensitized on days 0 and 14 by intraperitoneal (i.p.) injection of 100 μg OVA (S7951, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) emulsified in 1 mg aluminum hydroxide [Al(OH)$_3$; Pierce Chemical Co., Rockford, IL, USA] with a total volume of 0.2 ml. Following the last sensitization, mice were challenged wi-

th aerosolized 1% OVA at a total volume of 100 ml for 30 min each day between days 21-23. The control group was sensitized and challenged with PBS plus Al(OH)$_3$ and PBS, respectively. At 1 h before each challenge, the last three groups were given an additional intranasal administration of 100 μl (100 μg/mouse) PM2.5 solution at a concentration of 1 mg/ml [14], while the same volume of PBS was intranasally administered to the control and OVA groups at the same time point. Mice in the last two treatment-groups were injected with apigenin (i.p.; 42251, Sigma-Aldrich; Merck KGaA) and dexamethasone (Tianyao Company, Hubei, China), respectively. Apigenin (20 mg/kg/day), dissolved in 1% dimethyl sulfoxide (DMSO), (W387520, Sigma, St Louis, Mo, USA) was injected in a solution with a total volume of 200 μl between days 15-23. Mice in the other groups received 1% DMSO without apigenin. For the dexamethasone group, mice were injected with 5 mg/kg/day (i.p.) between days 15-23. The doses of apigenin and dexamethasone were based on our previous study [15, 17] (**Figure 2**).

Evaluation of airway responsiveness

Airway responsiveness to acetylcholine chloride (ACH) was measured in mice 24 h after the last challenge with an AniRes animal lung function analysis system (SYNOL High-Tech, Beijing, China). Following anesthesia by i.p. injection of pentobarbital sodium (70 mg/kg), a plastic tube was inserted into the trachea of mice for mechanical ventilation, and another into the caudal vein for drug administration. Mice were then placed in the whole-body plethysmography chamber and ventilated mechanically at a rate of 90 breaths/min with a tidal volume of 6 ml/kg. Following establishment of stable airway pressure, ACH was administered by the caudal vein with a microinfusion pump at a rate of 36 ml/h in progressively increasing doses (0, 10, 30, 90 and 270 μg/kg). Following the administration of each dose, data were collected continuously from 5 sec to 1 min and maximum values of lung resistance were recorded to express changes in AHR in the murine model.

Table 1. Metal and PAH composition in PM2.5

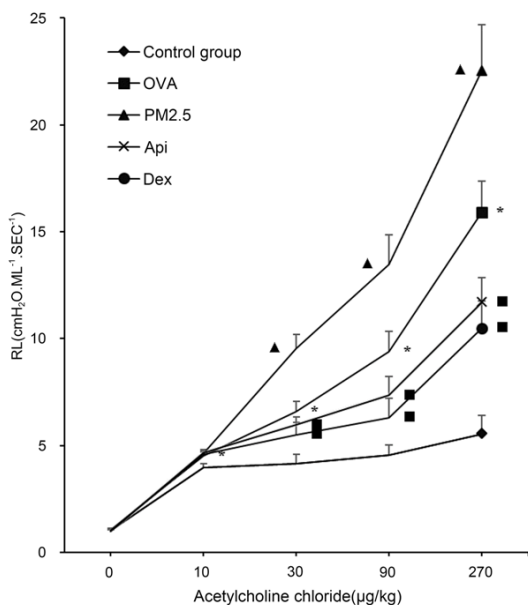| Metal	PM2.5 (µg/mg)	PAH	PM2.5 (µg/mg)
Na	60.51	Naphthalene	11.41
Mg	40.93	Acenaphthene	11.06
Al	37.28	Phenanthrene	9.17
Ca	32.17	Benzofluoranthene	4.69
Zn	18.93	Benzo	1.59
Fe	11.24	Fluoranthene	0.97
K	9.17	Pyrene	0.34
Mn	5.40	Chrysene	0.25
Ba	4.92	Anthracene	0.13
As	3.14		

Figure 3. Treatment with apigenin decreased the development of AHR in a murine model of asthma exposed to PM2.5. Data are the means ± SEM (n=6). *$P < 0.05$, compared with the control group. ▲$P < 0.05$, compared with the OVA group. ■$P < 0.05$, compared with the PM2.5 group.

Analysis of bronchoalveolar lavage fluid (BALF) and serum samples

Following assessment of AHR, mice were anesthetized, tracheas were inserted with a catheter, and airway lumina were washed three times with 0.4, 0.3 and 0.3 ml sterile saline. BALF was centrifuged, smears of BALF cells were stained with Wright's stain, and a minimum of 200 cells were counted in each of four random fields using a microscope. Then, mice were

sacrificed by cervical dislocation, blood samples were centrifuged, and serum was stored at -70°C until use. ELISA kits were used to determine the levels of total IgE (F-5330B, Jingmei, Shanghai, China) in serum, and of IL-4 (ab221833, Abcam, Cambridge, USA), IL-13 (ab-219634, Abcam) and IL-17 (ab199081, Abcam) in BALF.

Lung histology and periodic acid Schiff (PAS) staining

The right lung was removed for pathologic examination following lavage. The lung tissue was fixed in 10% formalin for 24 h. The specimens were then embedded and cut in a standard manner. The specimens were stained with hematoxylin and eosin (H&E) and then PAS stain to evaluate the level of inflammation and mucus production in the airways using Microsoft Image Pro Plus 6.0.

Measurement of total NF-κB protein by western blot

The left lung tissues from each group were added to lysis buffer and homogenized. Following absolute lysis and centrifugation at 12,000× g for 15 min at 4°C, the resulting supernatants containing total protein were collected. The protein concentration of samples was determined using the bicinchoninic acid method. The proteins were separated by 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane. The PVDF membrane was then incubated overnight with antibody against NF-κB p65 subunit (sc-514451, Santa Cruz, Biotechnology, USA) at 4°C. Subsequently, the membrane was incubated with secondary antibody (1:5,000) for 1 h at 37°C. The protein bands were detected with ECL Plus substrate. The level of target protein in each group was normalized to that of β-actin.

Statistical analysis

The data were expressed as the mean ± standard error. SPSS 22.0 (IBM, Corp., Armonk, NY, USA) was used to analyze the differences between experimental groups. Comparisons between measurements at two different times were performed using the paired *t*-test if the data were normally distributed. Otherwise, the Wilcoxon rank sum test was conducted. *P* <
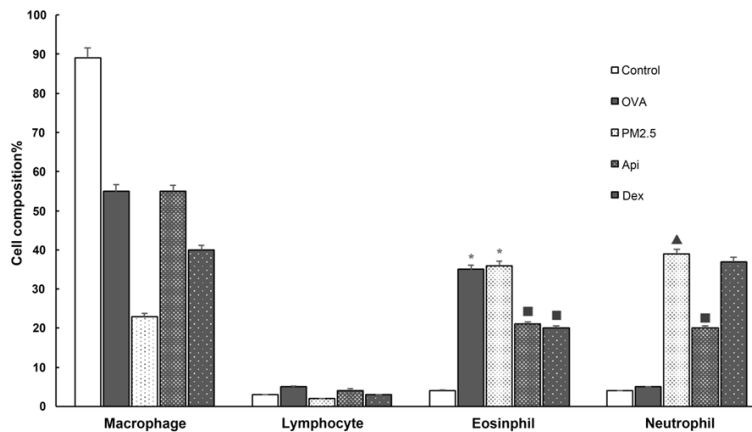
Figure 4. Treatment with apigenin inhibited both eosinophil and neutrophil accumulation in BALF. Data are the means ± SEM (n=6). *$P < 0.05$, compared with the control group. ▲$P < 0.05$, compared with the OVA group. ■$P < 0.05$, compared with the PM2.5 group.

0.05 or $P < 0.01$ was considered a significant difference.

Results

Analysis of the components of PM2.5

The chemical masses of constituents identified in the PM2.5 samples are listed in **Table 1**. Metals and PAHs comprised the main constituents of the ambient PM2.5 samples collected in Yantai. The results demonstrated that sodium, magnesium, aluminum, calcium, and zinc were the main metals, and naphthalene, acenaphthene, phenanthrene, benzofluoranthene, and benzopyrene the main PAHs in the ambient PM2.5, which may account for the exacerbating effect of PM2.5 on pre-existing asthma.

Apigenin reduces PM2.5 exposure-exacerbated AHR in asthmatic mice

To examine the effect of PM2.5 and apigenin on AHR, airway resistance was measured in anaesthetized mice by invasive whole-body plethysmography. No significant difference was identified in baseline airway resistance among the five groups. The airway resistance of the OVA group was notably increased compared with that of the control group following the administration of ACH at doses from 30 to 270 μg/kg; in turn, PM2.5 induced a marked increase in airway resistance compared with that in the OVA group (P < 0.05). Treatment with apigenin or dexamethasone resulted in a marked

decrease in airway resistance compared with that of the PM2.5 group (P < 0.05; **Figure 3**).

Apigenin attenuates PM2.5 exposure-induced inflammatory infiltration in the lungs of asthmatic mice

The effect of OVA, PM2.5 exposure, and apigenin on the airways and lungs of asthmatic mice was determined by counting cells in BALF and conducting lung histologic examination. Few inflammatory cells besides macrophages were detected in the BALF of control mice. A significant increase in the percentage of eosinophils was observed in OVA-sensitized and challenged animals compared with that in the control group (P < 0.05). Compared with that in the OVA mice, exposure to PM2.5 increased the percentage of neutrophils notably (P < 0.05), and thus led to a mixed inflammatory response. Therefore, PM2.5 exposure exacerbated the airway inflammation in asthmatic mice. The percentages of eosinophils and neutrophils in BALF were significantly decreased following administration of apigenin (P < 0.05). Treatment with dexamethasone also significantly decreased the percentage of eosinophils (P < 0.05), but exhibited less of an ability to decrease the number of neutrophils in asthmatic mice exposed to PM2.5 (**Figure 4**). The effects of OVA and PM2.5 were further demonstrated by histologic examination of H&E-stained samples. The results indicated notable inflammatory cell infiltration into the airways and around the blood vessels and focal infiltration of neutrophils in the PM2.5 group compared with the OVA group. Treatment with apigenin or dexamethasone significantly attenuated this inflammatory infiltration (**Figure 5A-F**). Additionally, mucus secretion was markedly increased in mice in the OVA and PM2.5 groups, especially particularly in the latter group. Treatment with apigenin and dexamethasone significantly attenuated this OVA and PM2.5-induced overproduction of mucus and goblet cell hyperplasia in mice, as determined from the percentages of PAS-positive cells (**Figure 6A-F**).
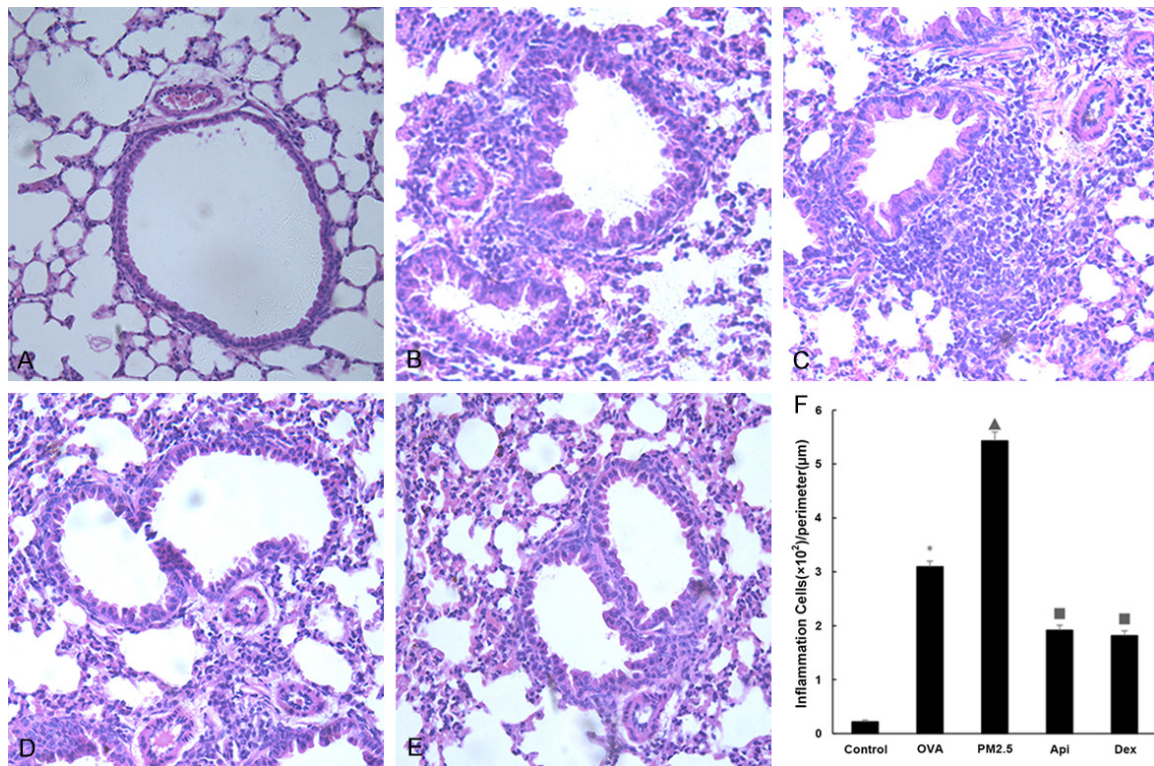
Figure 5. Treatment with apigenin attenuated airway inflammation in a murine model of asthma exposed to PM2.5. H&E staining (×200) of: (A) Comtrol group; (B) OVA group; (C) PM2.5 group; (D) Apigenin group; (E) Dexamethasone group; (F) Quantitative analysis of airway inflammation cells per perimeter.

Apigenin reduces PM2.5 exposure-enhanced levels of inflammatory cytokines in BALF and total IgE in serum

The concentrations of IL-4, IL-13, IL-17 and IgE were assessed by ELISA. The levels of Th2-related cytokines (IL-4, IL-13) and IL-17 in BALF and total serum IgE were significantly increased in the OVA and PM2.5 groups (P < 0.05). Exposure to PM2.5 significantly increased the levels of the BALF cytokines and serum IgE compared with those in the OVA group (P < 0.05). The administration of apigenin reduced the levels of BALF cytokines and total serum IgE (P < 0.05), while treatment with dexamethasone failed to decrease the level of IL-17 (P > 0.05). Thus, administration of apigenin attenuated the mixed Th2/IL-17 inflammatory response induced by exposure to PM2.5 in asthmatic mice (P < 0.05; **Figure 7**).

Apigenin alters PM2.5 exposure-enhanced expression of NF-κB p65 in the lungs

The relative expression of NF-κB p65 was quantified as the density of this target protein versus β-actin, and the relative density of total NF-κB p65 is presented as fold-change, particularly in the PM2.5 group (P < 0.05). In turn, treatment with apigenin or dexamethasone markedly decreased the expression of NF-κB p65 compared with that of asthmatic mice exposed to PM2.5 (P < 0.05; **Figure 8**).

Discussion

The present study, to our knowledge, was the first to investigate the effect of apigenin in mitigating the exacerbated AHR and airway inflammatory response induced by PM2.5 exposure in asthmatic mice. The study revealed that PM2.5 exposure increased OVA-induced inflammation and lung tissue injury, and induced a Th2-related response and IL-17-associated neutrophil infiltration in the lungs of asthmatic mice. The results suggest that patients with pre-existing asthma exposed to ambient PM2.5 may present with a mixed phenotype involving eosinophil and neutrophil infiltration. In the current study, the PM2.5 collected in Yantai contained high concentrations of metal and PAH elements. Keiki *et al.* [22] reported that PM2.5
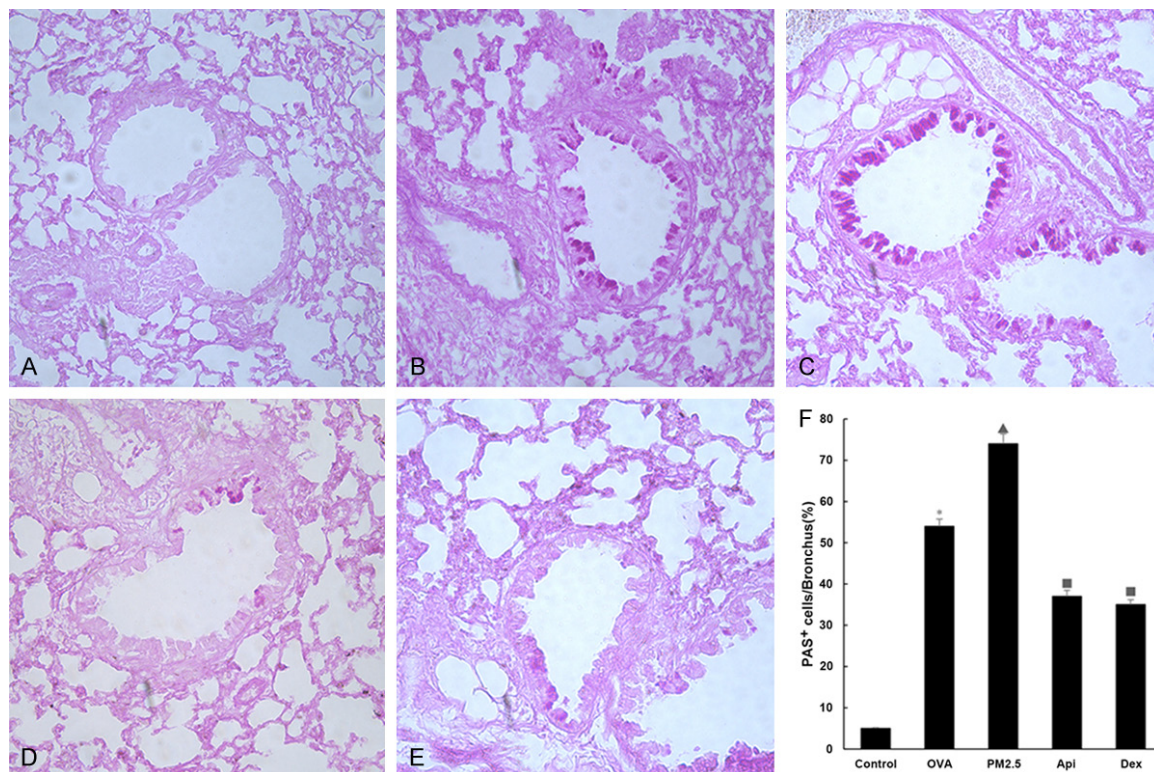
Figure 6. Treatment with apigenin attenuated mucus production in a murine model of asthma exposed to PM2.5. PAS staining (×200) of: (A) Comtrol group; (B) OVA group; (C) PM2.5 group; (D) Apigenin group; (E) Dexamethasone group. (F) The percentage of PAS-positive cells per bronchiole. Data are the means ± SEM (n=6). *$P < 0.05$, compared with the control group. ▲$P < 0.05$, compared with the OVA group. ■$P < 0.05$, compared with the PM2.5 group.

containing higher concentrations of metals induced AHR and eosinophil infiltration in mice with genetically predisposed high sensitivity to mite allergens; another study determined that several metals including zinc, lead, copper and cadmium were associated with the effect of PM2.5 on airway inflammation and increased Th2-related cytokines in OVA-allergic mice [23]. Classic asthma is characterized by Th2 airway inflammation, involving high levels of eosinophils, total IgE, and Th2-related cytokines. Meanwhile, the present study also demonstrated that PM2.5 increased the production of IL-17. A previous study reported a strong correlation between the levels of IL-17 and neutrophils in blood and sputum [24]. The levels of IL-17 and Th17 cells were also positively correlated with asthma severity in a previous asthmatic cohort [8]. Deficiency of IL-17 has been suggested to lead to lack of a neutrophilic response and AHR to allergens [25]. IL-17 has also been reported to induce steroid resistance in peripheral mononuclear cells [26]. Other studies have described the role of aryl hydrocarbon receptor, a primary receptor for PAHs, in the regulation of Th17 differentiation [27, 28]. Thus, the exposure of asthmatic mice to PM2.5 in the current research induced notable increases in AHR, eosinophil, and neutrophil infiltration, and Th2 cytokines and IL-17, which may be attributed to the high concentrations of metals and PAHs in the ambient PM2.5 samples. In addition, NF-κB has been reported to be involved in the pro-inflammatory response and to be activated in asthma, particularly in severe subtypes. p65 protein is the key component of NF-κB. Hart *et al.* [29] reported that NF-κB p65 activation was associated with inflammation in the airways of asthmatics. In the present study, the expression of NF-κB p65 in the PM2.5 group was higher than that in the OVA group, suggesting that PM2.5 may enhance the airway inflammation of asthmatic mice through influencing the activation of NF-κB p65.

The protective effect of flavonoids in inflammatory disorders has gained attention worldwide.
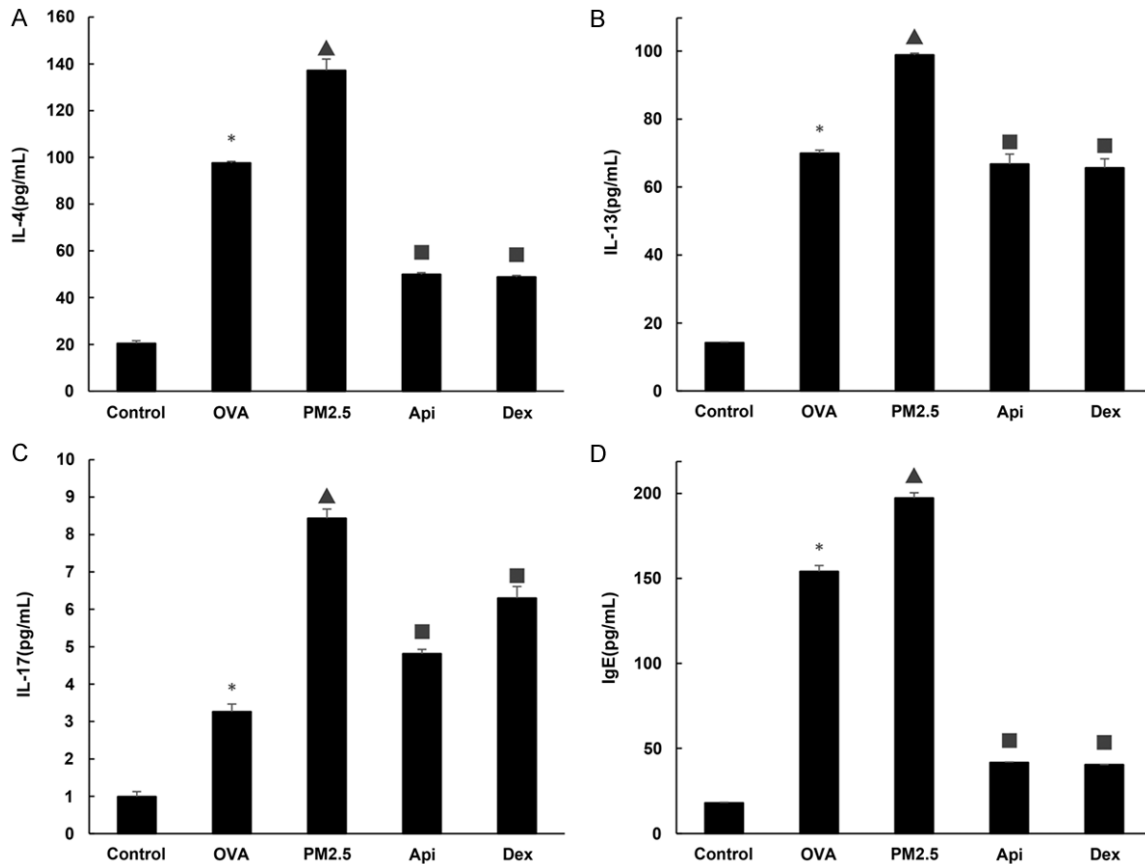
Figure 7. Treatment with apigenin reduced the levels of Th2-type cytokines IL-4, and IL-13 in BALF and total serum IgE in a murine model of asthma exposed to PM2.5. Data are the means ± SEM (n=6). *$P < 0.05$, compared with the control group. ▲$P < 0.05$, compared with the OVA group. ■$P < 0.05$, compared with the PM2.5 group.
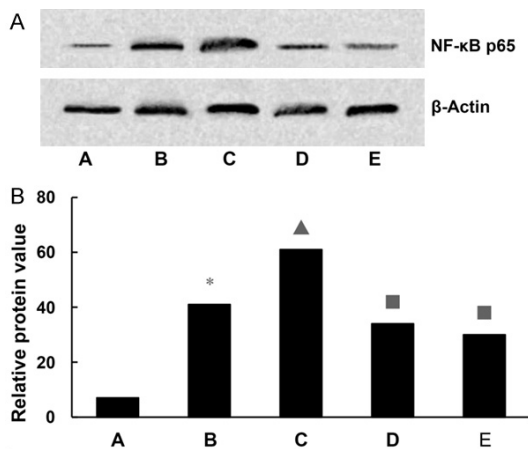

Figure 8. Treatment with apigenin decreased the level of NF-κB p65 in the lung in a murine model of asthma exposed to PM2.5. A. control group; B. OVA group; C. PM2.5 group; D. apigenin group; E. dexamethasone group. Data are the means ± SEM (n=6). *$P < 0.05$, compared with the control group. ▲$P < 0.05$, compared with the OVA group. ■$P < 0.05$, compared with the PM2.5 group.

Luteolin had been reported to inhibit IL-6 and TNF-α release by suppressing NF-κB activity in human monocytes stimulated with lipopolysaccharide [30]. Supplementation of quercetin to human subjects significantly decreased the serum concentration of TNF-α [31]. Naringenin has also been suggested to prevent airway remodeling by reducing the production of reactive oxygen species in asthmatic mice [15]. A previous study by our group only assessed the beneficial effects of apigenin on an OVA-induced mouse model of asthma. According to the present data, administration of apigenin significantly attenuated AHR, airway inflammation, and the mixed Th2 and IL-17 response induced by PM2.5 and OVA co-exposure. The pathophysiology of AHR is complex, with many factors contributing to its development. Th2 cytokines, particularly IL-4 and IL-13, are critical in allergic airway inflammation and the development of AHR [32]. In the present study, airway resistance in mice treated with apigenin

was decreased following a challenge with ACH, in accordance with the changes in the percentage of inflammatory cells and Th2 cytokine levels in BALF. Th17 cells are considered to be involved in steroid-resistant neutrophilic airway inflammation [33]. Dexamethasone exhibited less ability to decrease neutrophils and the level of Th17 in asthmatic mice exposed to PM2.5 in the current study. Meanwhile, apigenin decreased the infiltration of neutrophils and the expression of IL-17, and was suggested to influence the differentiation of Th17 cells. However, the expression of NF-κB p65 was decreased in both the apigenin and dexamethasone treatment groups; thus the NF-κB pathway was indicated to be involved in the effect of apigenin treatment in asthmatic mice exposed to PM2.5, and we cannot exclude another unknown pathway, which provides a direction for follow-up research.

In conclusion, apigenin exhibited significant efficacy in preventing the exacerbation of a lung inflammatory response induced by PM2.5 exposure in a murine model of pre-existing asthma by down-regulating NF-κB. This suggests that apigenin may be promising as a medication for the treatment of asthma, particularly for cases of severe asthma. NF-κB p65 only cannot represent the whole activity of the NF-κB pathway, which was a limitation of our study. However, the present study provided theoretical basis for further signal pathway mechanism study.

Disclosure of conflict of interest

None.

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