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Protective Effects of Ferulic Acid on Respiratory Injuries in PM2.5-Induced Asthmatic Rats

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Abstract

Ferulic acid (FA) has been reported to have anti-inflammatory effects. However, the protective of FA on respiratory injuries in PM2.5-induced asthmatic rats remain unclear. This study was to investigate the effects of FA on respiratory injuries in $PM_{2.5}$-induced asthmatic rats. Asthmatic rat models were established with high dose sensitization, inhaled with ovalbumin (OVA) by intratracheal instillated and pulverization. The PM2.5 was intratracheal instilled for three times. FA was pretreated by oral administration. The lung tissues were observed by HE staining. The number of inflammatory cells in bronchoalveolar lavage fluid (BALF) were counted and the levels of inflammatory mediators transforming growth factor (TGF)-β1, heparin-binding epidermal growth factor(HB-EGF), interleukin(IL)-10 and IL-17 in BALF were detected. The levels of IFN-γ, IL-4, IL-5, tumor necrosis factor(TNF)-α, macrophage inflammatory protein (MIP)-1α, eosinophil chemotactic factor (eotaxin)-1, matrix metalloproteinases (MMP)-9, tissue inhibitor of metalloproteinases inhibitor(TIMP)-1, IL-8 and the activity of acidic phosphatase(ACP), alkaline phosphatase(AKP), lactate dehydrogenase(LDH) in serum were detected. The concentrations of OVA-specific immunoglobulin E (OVA-sIgE) and IgE in lung tissue were detected. All the cytokines were tested by Enzyme-Linked Immunosorbnent Assay (ELISA). Histopathological analyses showed that 19.4 mg/kg and 38.8 mg/kg FA had protective effects against PM2.5-induced lung inflammation. The levels of IL-4, IL-5, TNF-α, MIP-1α, Eotaxin-1, MMP-9, TIMP-1, IL-8, IgE, OVA-sIgE, TGF-β1, HB-EGF and IL-17 and the activities of ACP, AKP and LDH were also inhibited , and the levels of IFN-γ and IL-10 were up-regulated. In conclusion, these data suggest that FA demonstrated potential protective effects against the respiratory injuries aggravated by PM2.5 in asthmatic rats. These protective effects may be achieved by ameliorating the airway inflammatory response and regulating the imbalance of Th1/Th2 and Th17/Treg.

Keywords: Asthma; PM2.5; Ferulic acid; Respiratory injuries; Th1/Th2; Th17/Treg

Introduction

Asthma is a major chronic airway inflammatory disease characterized by airways hyperresponsiveness (AHR), reversible airflow obstruction, airway remodeling, and episodic exacerbations caused by air pollutants, such as particulate matter (PM; PM <2.5 nm in diameter [PM2.5]) [1], and its prevalence and mortality have been increasing rapidly in recent years. The world's allergic asthma accounts for about 20% of the world's population [2]. At present, the proposed pathogenesis of asthma mainly includes inflammatory cell infiltrates, changes in the signal transduction pathways, the imbalance of helper T cell (Th)1/Th2 and Th17/regulatory T cell (Treg). Inflammation in asthma consists of airway infiltration by Mastocytess, lymphocytes, and eosinophils. Eosinophilic inflammation of the airway is correlated with the severity of asthma [3]. The production of IL-4 and IL-10 by Th2 cells blocks the production of cytokines by Th1 and NK cells. Th1 cells, by secreting interferon-γ (IFN-γ), inhibit the proliferation and differentiation of basophils, mastocytes, and eosinophils, whose activities are controlled for by IL-3, IL-4, IL-5, and IL-10 from Th2 cells. However, it has recognized that Th1/Th2 imbalance does not fully explain the pathogenesis of asthma in recent years. Some studies suggest that the imbalances in Th17/Treg cells and Th1/Th2 cells play an important role in asthma [4-6]. An imbalance of Th1/Th2 cells may initiate allergic asthma, while an imbalance of Th17/Treg cells is associated with worsening of allergic asthma [7]. Further studies are needed to clarify the relationship between Th1/Th2 and Th17/Treg, and assess how these cells play a more important role in allergic asthma. Moreover, the chemokines such as Eotaxin-1, IL-8 and macrophage inflammatory protein (MIP)-1α also participate in the occurrence and development of asthma. Matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of metalloproteinase-1 (TIMP-1) are also involved in inflammatory and fibrogenic processes, though the roles that these cytokines play in asthma are not absolutely clear.

PM2.5 can cause damage to many organs in human body, especially the respiratory, cardiovascular system, resulting in the development and exacerbation of bronchial asthma, lung cancer, and cardiovascular diseases. Animal experiments showed that tracheal instillation of PM2.5 can cause or induce acute or chronic respiratory tract inflammation and allergic reactions in OVA-induced asthmatic rats or mice [8,9] PM2.5 can lead to increases in IL-4, IL-5, IL-10 levels, and affect the immune response mediated by Th2 [9]. PM2.5 can induce both local and systemic inflammatory responses in mice, cause the secretion disorder of cytokines, such as IFN-γ, IL-4, IL-5 and IL-10, etc, and lead to an imbalance in the Th1/Th2 pathway, especially the Th2 pathway [10]. Exposure to PM2.5 for a long time can aggravate or prolong inflammatory reaction and cause the imbalance of Th17/Treg cells and related cytokines [11]. However, there are few studies on PM2.5-induced asthma in rats and the mechanism of PM2.5 on airway inflammation is unclear.

Ferulic acid (FA) belongs to the family of phenolic acids and widely exists in vegetables, fruits, and some beverages such as coffee and beer [12]. It has many therapeutic effects. Phenolics in the juice afforded protection against induced oxidative stress, most probably by means of an iron-chelating mechanism [13]. FA anti-inflammatory effects are mainly mediated through IKK/NF-κB signaling pathway [14]. FA has the analgesic effect. Its mechanism may be related to the inhibition of monoamine oxidase activity and the increase in monoamine neurotransmitter in mouse [15].It can also protect target organs. FA prevents diosbulbin B-induced liver injury via ameliorating diosbulbin B-induced liver oxidative stress injury and augments diosbulbin B-induced anti-tumor activity [7]. FA also has certain protective effects on the nerve injury of cerebral ischemia, and suggest that promoting erythropoietin in the brain and peripheral blood may be one of the neuroprotective mechanisms of FA [16]. An *in vitro* study showed that FA could reduce oxidative stress, inhibit the PI3K/Akt and NF-κB pathway, and down-regulate the expression of pro-apoptotic protein (BCL-2/BCL-XL-associated death promoter) in injuried-cells caused by PM2.5 [17]. FA has an anti-allergic effect via restoring Th1/Th2 imbalance by modulating dendritic cells (DCs) function in an asthmatic mouse model[18]. However, the effect of FA on PM2.5-induced asthma rats and its protective mechanism have not been reported.

Therefore, the changes in cytokines (such as IL-4, IL-5, IFN- λ, and so on) which could affect the balance of Th1/Th2 and Th17/Treg

at the molecular level were investigated in the study to clarify whether the damage effects of PM2.5 on asthmatic rats can be reduced by FA. It is of great significance to further clarify the pathogenesis of severe asthma and explore the potential targets for asthma treatment.

Materials and Methods

Animals

Male SD rats of 160-180g weight were purchased from the Beijing Weitong Lihua Experimental Animal Technology Co.Ltd (approval number: SCXK (Beijing) 2012-0001) and housed in the Function Food Testing Center of College of Arts and Sciences of Beijing Union University (approval number: SYXK (Beijing) 2014-0031). Basic feed was produced by Beijing Hua Fukang biological Polytron Technologies Inc. (approval number: SCXK (Beijing) 2014-0030). Rats were housed in 4 per cage with free access to food and water. The environment was set on a 12h dark-light cycle at a temperature level of 23 ± 2°C.

Collection and suspension preparation of PM2.5

From December 2015 to February 2016 (heating season), PM2.5 samples were collected on quartz filters using the TH-1000 TSP high volume sampler (Wuhan Tianhong Instrument Co., Ltd, Wuhan), which was placed on the rooftop of a building with a height of 9m beside the Xueyuan Road of Beijing City and there were no large obstacles near the building. The sampler was set at 78m^3/h flow rate for a period of 22h for each measurement. The filters were cut into 6 cm^2 small pieces and then put into sterilized beaker with 50 ml sterilized pure water, sonicated for 1 min and were then filtrated with sixfold sterilized gauze to obtain PM2.5 extracts which were freeze-dried in vacuum for 24 h and stored at -20°C. Prior to use, the dried samples were diluted with sterilized phosphate-buffered saline solutions (PBS, pH 7.2) and sonicated for 15 min to disperse possible aggregates.

Reagents

MK360 quartz filters were purchased from Beijing Safelab Technology company (Beijing, China). Ovalbumin (OVA) and dexamethasone purchased were from Sigma (USA). Chloral hydrate and FA (Purity: 99%,CAS:1135-24-6,) were purchased from Aladdin(Shanghai). Wright Giemsa dye were purchased from solarbio science and technology (Beijing, China). Rat assay kits for ACP, AKP and LDH were purchased from Nanjing Jiancheng Biotechnology Company (Nanjing, China). Rat enzyme linked immunosorbent assay (ELISA) kits for IL-4, IL-5, TNF-α, MIP-1α, Eotaxin-1, MMP-9, TIMP-1, IL-8, TGF-β1, HB-EGF, IL-17, IgE, OVA-sIgE, IFN-γ and IL-10 were purchased from Shanghai Hushang Biotechnology Company (Shanghai, China).

Animal models and drug administration

Figure 1 schematically depicts the experimental protocol used in this study. Fifty-six male SD rats were randomly assigned into the following seven groups (n = 8 for each group): control group, model (OVA) group, PM2.5 (OVA+6.0 mg/kgPM2.5) group, FA (19.4) group (OVA+6.0 mg/kg PM2.5+19.4mg/kg FA), FA (38.8) group (OVA+6.0 mg/kg PM2.5+38.8mg/kg FA), FA (77.6) group (OVA+6.0 mg/kg PM2.5+77.6mg/kg FA) and positive group (OVA+6.0 mg/kg PM2.5+dexamethasone).The dosage of FA is 5, 10 and 20 times of the recommended dosage of human body. OVA-sensitized rats were used to build the asthma models. Rats were exposed to OVA for sensitization and challenge, while rats in the control group were sensitized and challenged only using saline. On 1st day, 14th day and 21th day, 1 mL suspension containing 2mg of freshly prepared OVA and 10% $Al(OH)_3$ in sterile saline were injected into medial subcutaneous of four limbs and intraperitoneal medial subcutaneous(s.c.), with 0.2 mL for each point. Simultaneously 0.2 mL adjuvant, Bordetella pertussis vaccine containing 5×10^9 heat-killed bacteria was administered intraperitoneally. On 28th day, Rats were given 1.7% OVA via intratracheal instillation (i.n.) for sensitization, and then placed in a transparent container (20 liters), and sprayed with 1% solution of OVA for 30 min using ultrasonic nebulizer (402AI, YuYue Company Ltd., Jiangsu, China) to challenge asthma once per day for one week continuously.

From the first day of the experiment, different doses of FA or saline or dexamethasone (0.2mg/kg) were given orally to rats for 28 days continuously with gavage capacity of 5ml/kg. At 29th day, rats were hocused with 5mg/kg chloral hydrate, exposed to PM2.5 or saline by tracheal instillation for three times, then ultrasonic atomizing inhalation for 30 min with 1% OVA for 5 consecutive days. After the last exposure to PM2.5 within 24 hours, the rats were anesthetized by intraperitoneal injection of chloral hydrate (0.3mg/kg bw). Then the femoral artery blood, BALF and the lung tissues of the rats were collected respectively (Figure 1).

Collection and analysis of blood samples

Within 24 h after the final challenge, the rats were were sacrificed to get the samples. Serum samples were then collected from femoral vein blood by centrifugation (3000 rpm at 24°C for 15 min) and stored at -80°C. Serum IFN- γ, IL-4, IL-5, TNF- α, MIP-1α, Eotaxin-1, MMP-9, TIMP-1, IL-8 concentrations were determined by ELISA kits using an ELISA reader. ACP, AKP and LDH activities were determined by Trace Enzyme Standard Method. Assays were performed as recommended by the manufacturers.

Collection and analysis of BALF

To obtain BALF, the left lungs were washed three times with PBS. The amount of lung lavage was measured according to 35 ml/kg × rat weight (kg) × 0.4 (total lung capacity of each rat× left lung total lung volume 40%). BALF was centrifuged at 4°C 1500 rpm for 10 min. Cell precipitation was adjusted to 1×10^6 cells /ml, and then cells were smeared and dried after Giemsa staining. At least 200 cells were counted and identified as eosinophils, neutrophils, or lymphocytes under light microscope with × 200 magnification. At the same time, the

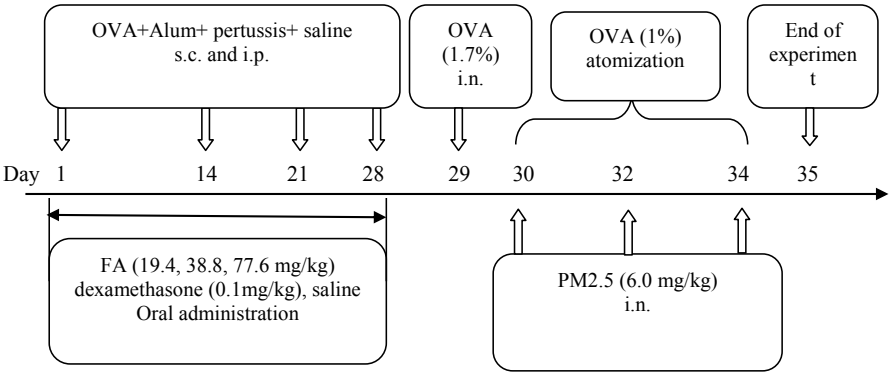


Figure 1: Animal models and drug administration.

supernatant of BALF was centrifuged at 4°C 3000 rpm for 10 min and then the supernatant was used as the determination of TGF-β1, HB-EGF, IL-10 and IL-17 by ELISA which were performed according to the manufacturer's instructions.

Preparation of lung tissue homogenate

The rats were sacrificed by cervical dislocation and the right lung was removed by medical scissors and rinsed in ice-cold PBS. Lung tissue was homogenized in a glass homogenizer, using 10 ml/g of ice-cold PBS at pH 7.5 to produce a 10% tissue homogenate. Half of the homogenate was centrifuged at 10,000 rpm and 4°C for 10 min and the supernatant was collected and frozen at -80°C for later assay of the IgE and OVA-sIgE by ELISA kits. The remaining lungs were fixed with 10% neutral formalin, embedded in paraffin, sectioned at a thickness of 4 μm and stained with haematoxylin and eosin (H&E) for histopathology [19] and the damage of lung tissue and the infiltration of inflammatory cells were observed by light microscopy.

Statistical analysis

Experimental results were shown as mean ± standard error of the mean (SEM). SPSS 19 and Origin 9.1 softwares were used for data processing. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Bonferroni test. A value of <0.05 (2-sided) was considered to be statistically significant.

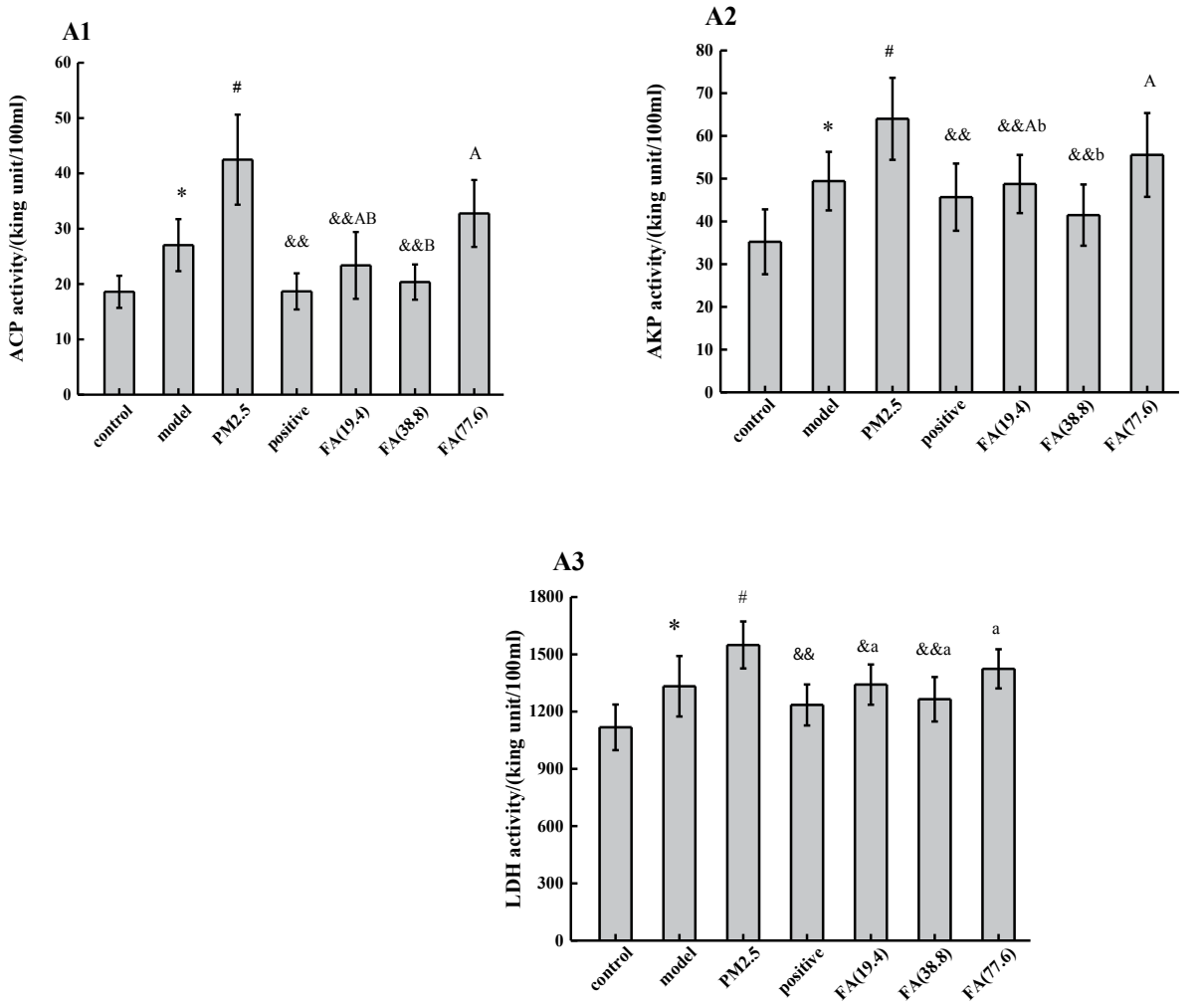
Results

Effect of FA on cytotoxicity induced by PM2.5 in asthmatic rats

To estimate the level of cytotoxicity induced by PM2.5 and the protection of FA, ACP, AKP and LDH activities were measured (Figure 2A1-A3). Results showed that ACP, AKP and LDH activities in the model group was significantly increased compared with the control group. Activities of these markers were also increased in the PM2.5 group compared with the model group. Moreover, FA (19.4) and FA (38.8) could decrease ACP, AKP and LDH activities induced by PM2.5. These results suggest that PM2.5 could aggravate lung toxicity in asthmatic rats, and FA (19.4mg/kg, 38.8mg/kg) could antagonize the toxic effects induced by PM2.5.

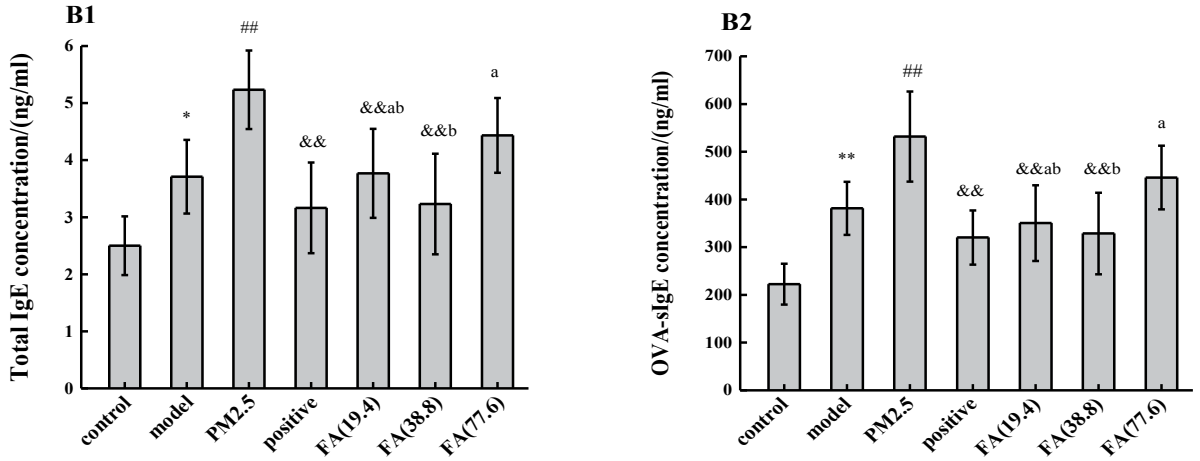
Effect of FA on lung Ig concentrations induced by PM2.5 in asthmatic rats

Total IgE and OVA-sIgE were two indicators of allergic asthma. As shown in Figure 3B1 and 3B2, OVA challenge resulted in marked increases of total IgE and OVA-sIgE in asthmatic model group compared with control group and dexamethasone (0.2mg/kg) caused significant reductions in these two indicators in the positive group. The productions of total IgE and OVA-sIgE were further increased after 6.0 mg/kg PM2.5 treatment in the presence of OVA compared with the model (OVA) group. However, the levels of total



Note: (1) Note: compared with the control group, *p<0.05, **p<0.01; compared with the model group, #p<0.05, ##p<0.01; compared with the PM2.5 group, &p<0.05, &&p<0.01; compared among the FA groups, with different letters represent p<0.05, with different Capitals represent p<0.01,different letters or Capitals represent the difference.(2) A1,A2,A3 are the activity of ACP, AKP, LDH, respectively.

Figure 2: Effect of FA on the activities of ACP, AKP, LDH induced by PM2.5 in asthmatic rats.



Note: compared with the control group, *p<0.05, **p<0.01; compared with the model group, #p<0.05, ##p<0.01; compared with the PM2.5 group, &p<0.05, &&p<0.01; compared among the FA groups, with different letters represent p<0.05, different letters represent the difference.

Figure 3: Effect of FA on lung Ig concentrations induced by PM2.5 in asthmatic rats.

Table 1: Effect of FA on inflammatory cell recruitment induced by PM2.5 in asthmatic rats.

Group	Total cells (1×10^6个/ml)	Differential leukocyte count (%)		
		Eosinophils	Neutrophils	Lymphocytes
control	$0.70 \pm 0.120 \pm 0.12^{**}$	$1.88 \pm 0.58^{**}$	$1.44 \pm 0.42^{**}$	$0.75 \pm 0.71^{**}$
model	$1.50 \pm 0.12^{\#\#}$	$7.8 \pm 1.02^{\#\#}$	$3.13 \pm 0.69^{\#}$	$3.00 \pm 0.80^{\#}$
$PM_{2.5}$	$3.01 \pm 0.20^{\&\&}$	$11.72 \pm 1.97^{\&\&}$	$5.63 \pm 1.48^{\&\&}$	$5.87 \pm 1.66^{\&\&}$
positive	$1.03 \pm 0.09^{\&\&b}$	$4.76 \pm 1.19^{\&\&b}$	$2.19 \pm 0.59^{\&b}$	$1.88 \pm 0.64^{\&ab}$
FA (19.4)	$1.45 \pm 0.10^{\&\&b}$	$6.48 \pm 1.27^{\&\&b}$	$2.88 \pm 0.35^{\&\&b}$	$2.81 \pm 0.70^{\&\&b}$
FA (38.8)	$1.42 \pm 0.12^{\&\&A}$	4.94 ± 1.08	2.43 ± 0.42	2.25 ± 0.65^{a}
FA (77.6)	2.18 ± 0.24	$8.93 \pm 1.24^{\&\&A}$	4.56 ± 0.86^{A}	4.63 ± 1.41

Note: compared with the control group, *p<0.05, **p<0.01; compared with the model group, #p<0.05, ##p<0.01; compared with the PM2.5 group, &p<0.05, &&p<0.01; compared among the FA groups, with different letters represent p<0.05, with different Capitals represent p<0.01, different letters or Capitals represent the difference.

IgE and OVA-sIgE were decreased in the FA groups compared with the PM2.5 group.

Effect of FA on inflammatory cell proliferation induced by PM2.5 in asthmatic rats

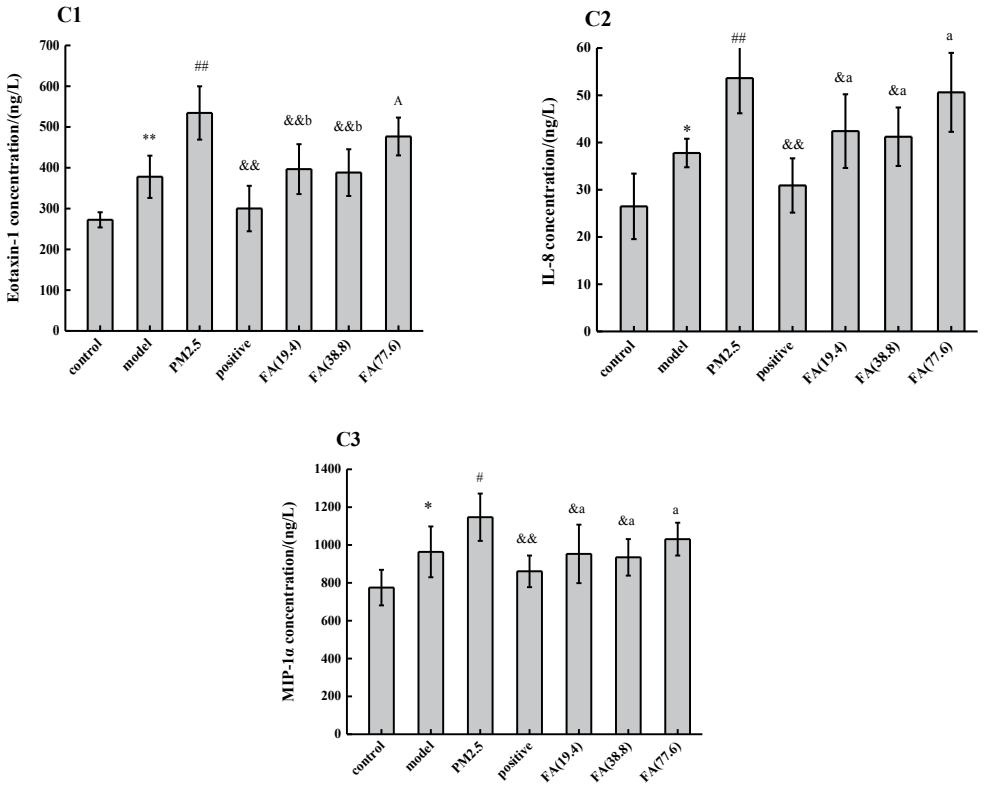
Effect of FA on inflammatory cell recruitment induced by PM2.5 in asthmatic rats: The changes in the BALF cellular profile showed that the numbers of total cells, eosinophils, neutrophils and lymphocytes were significantly increased in the model group compared with the control group, and it was reduced in the positive group compared with the PM2.5 group (Table 1). The differential leukocyte counts in the PM2.5 group were greater than those in the model group, suggesting that PM2.5 could aggravate the injury in asthmatic rats by increasing the numbers of inflammatory cells. Compared with the PM2.5 group, the numbers of total cells, eosinophils, neutrophils and lymphocytes were substantially decreased in FA groups except neutrophils and lymphocytes at FA (77.6 mg/kg) group. Therefore, FA in high dose had poor protective effects to antagonize the effect of PM2.5 in asthmatic rats to some extent, indicating that the interventional effect of FA was related to its dosage. The change in eosinophils was more substantial than the changes in neutrophils and lymphocytes.

Effect of FA on the chemokine levels induced by PM2.5 in asthmatic rats: To estimate the effect on chemokines induced by PM2.5 and the protection of FA in asthmatic rats, Eotaxin-1, IL-8 and MIP-1α concentrations were measured (Figure 4C1-4C3). Results showed that Eotaxin-1, IL-8 and MIP-1α levels were increased in the model group compared with the control group and were further increased in the PM2.5 group compared with the model group. 19.4 mg/kg FA and 38.8mg/kg FA could decrease the levels of Eotaxin-1, IL-8 and MIP-1α. Therefore, PM2.5 could increase the secretion of chemotaxis factors which may aggravate the development of asthma. FA (19.4mg/kg, 38.8mg/kg) could antagonize the effects of PM2.5 on asthma rats by regulating chemokine secretion disorder.

Effect of FA on the imbalance of MMP-9 /TIMP-1 induced by PM2.5 in asthmatic rats: To study the effect of FA on the imbalance of MMP-9 /TIMP-1 in PM2.5-exposed asthmatic rats, the levels of MMP-9 and TIMP-1 were detected and the ratio between them was calculated (Figure 5D1-5D3). Results showed that MMP-9 and TIMP-1 levels in the model group were significantly increased compared with the control group. Notably, 6.0mg/kg PM2.5 could increase the secretion of MMP-9 and TIMP-1 in asthmatic rats (Figure 5D1 and D2). However, the ratios of MMP-9 toTIMP-1 had no significant difference between different groups (Figure 5D3). Furthermore, FA (19.4) and FA (38.8) could significantly decrease MMP-9 and TIMP-1 levels.

Effect of FA on the imbalance of Th1/Th2 induced by PM2.5 in asthmatic rats

IFN-γ (a Th1 cytokine), IL-4 and IL-5 (Th2 cytokines) were assayed to verify the presence of airway inflammation in rat asthma model. As shown in Figure 6E1-6E5, model group displayed decreases in IFN-γ contents and increases in IL-4, IL-5 and TNF-α concentrations compared with the control group. The ratios of IFN-γ to IL-4 and IFN-γ to IL-5 were also lower, suggesting that the Th1/Th2 balance was broken and the Th2 response played a dominant role in OVA-treated rats in model group. 6.0 mg/kg PM2.5 facilitated Th2 immune responses by inducing increases in the levels of IL-4, IL-5 and TNF-α and decreases in the levels of IFN-γ, IFN-γ/IL-4 in the presence of OVA, compared with the model groups. What's more, treatments with



Note: (1) Note: compared with the control group, *p<0.05, **p<0.01; compared with the model group, #p<0.05, ##p<0.01; compared with the PM2.5 group, &p<0.05, &&p<0.01; compared among the FA groups, with different letters represent p<0.05, with different Capitals represent p<0.01, different letters or Capitals represent the difference. (2) C1,C2,C3 are the contents of Eotaxin-1, IL-8 and MIP-1α respectively.

Figure 4: Effect of FA on Eotaxin-1, IL-8 and MIP-1α concentrations induced by PM2.5 in asthmatic rats.

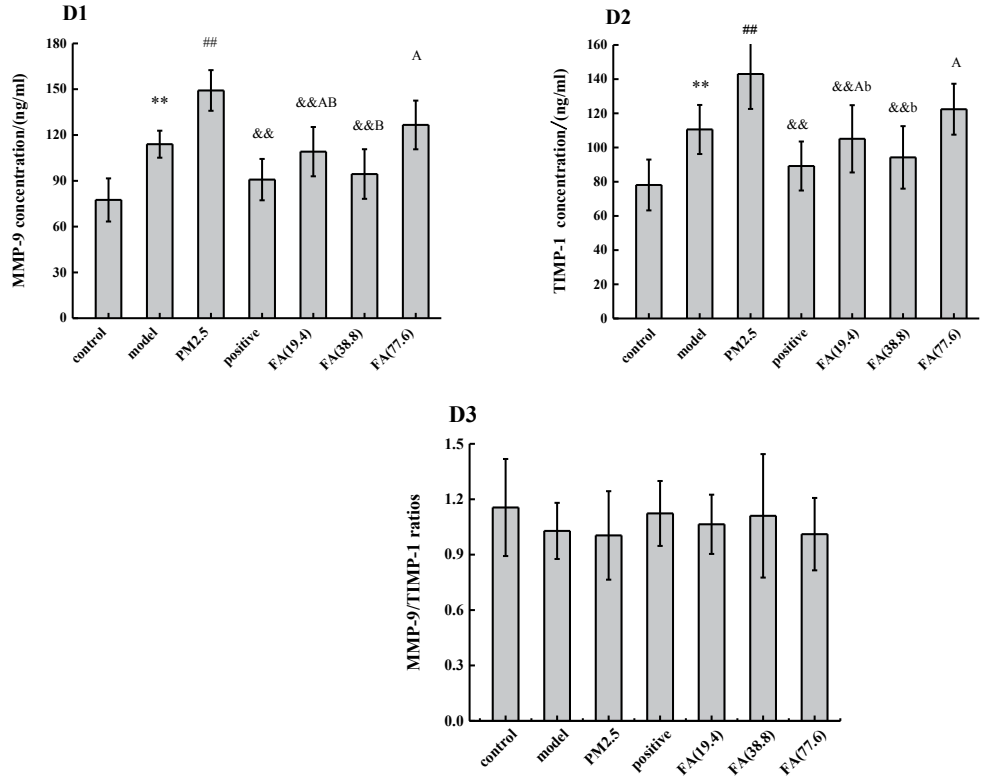


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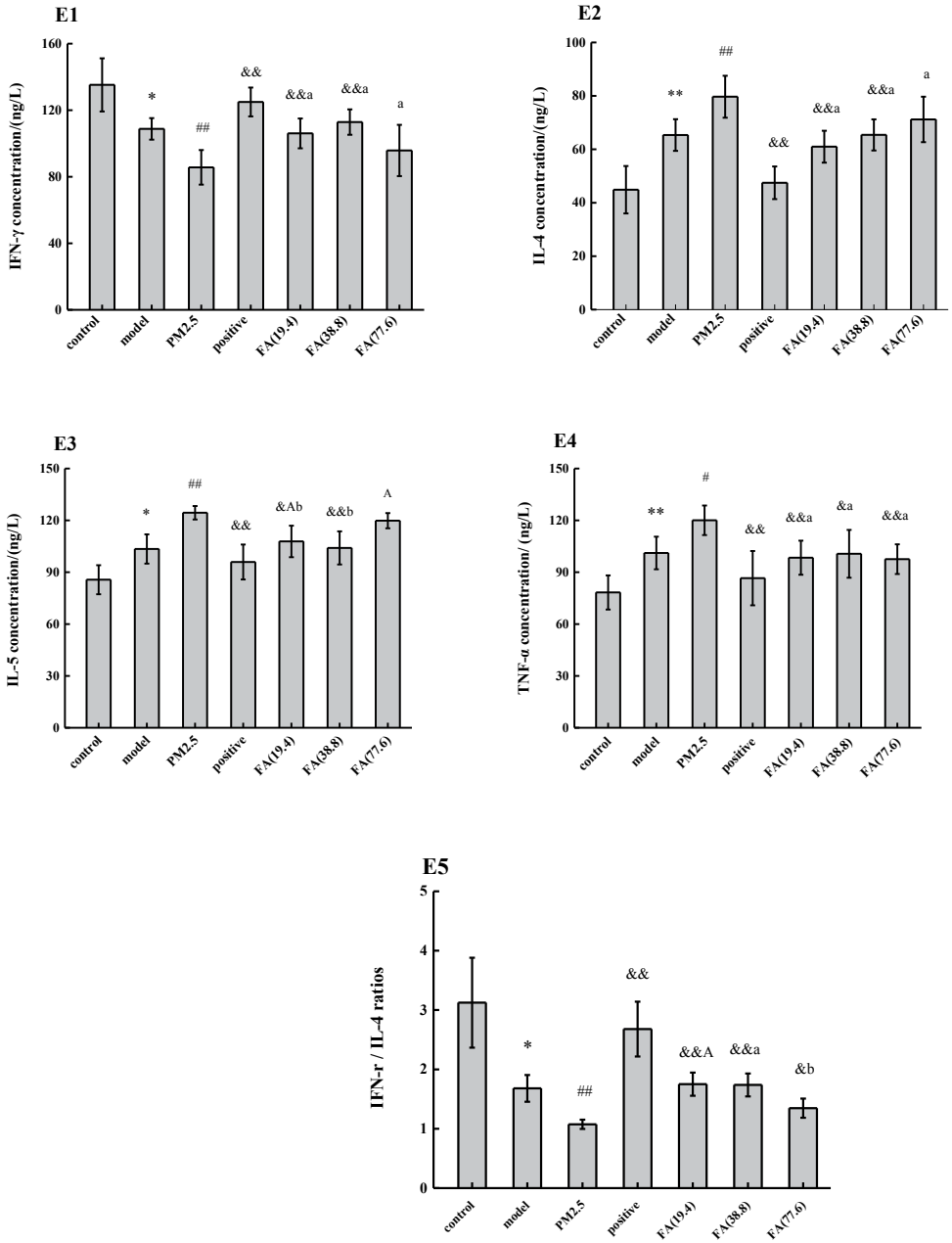
Figure 5: Effect of FA on the imbalance of MMP-9/TIMP-1 induced by PM2.5 in asthmatic rats.



Note: (1) Note: compared with the control group, *p<0.05, **p<0.01; compared with the model group, #p<0.05, ##p<0.01; compared with the PM2.5 group, &p<0.05, &&p<0.01; compared among the FA groups, with different letters represent p<0.05, with different Capitals represent p<0.01, different letters or Capitals represent the difference. (2) E1-E4 are the contents of IFN-γ, IL-4, IL-5, TNF- α, and B5, E5 is the ratios of IFN-γ to IL-4.

Figure 6: Effect of FA on the imbalance of Th1/Th2 induced by PM2.5 in asthmatic rats.

FA (19.4mg/kg, 38.8mg/kg) could partially restore the imbalance of Th1 and Th2 immune response induced by PM2.5.

Effect of FA on the imbalance of Th17/Treg induced by PM2.5 in asthmatic rats

IL-17 (a Th17 cytokine) and HB-EGF (a downstream molecule of IL-17) together with IL-10 and TGF-β 1 (Treg cytokines) were assayed to verify the effect of PM2.5 and FA on the imbalance of Th17/Treg in asthmatic rats (Figure 7F1-7F5). HB-EGF is an indicator for the severity of asthma The model group showed declines in IL-10 and increases in HB-EGF, IL-17 and TGF-β1 compared with the control group. 6.0 mg/kg PM2.5 could decrease IL-10 and increase HB-EGF, IL-17 and TGF-β1 compared with the model group. In all the FA groups except the FA (77.6 mg/kg) group, the levels of HB-EGF, IL-17 and TGF-β1 were increased and the level of IL-10 was decreased compared with the PM2.5 group. The ratio of IL-17 to IL-10 was also increased, suggesting that the balance of Th17/Treg was restored and the Th17 response played a dominant role. Therefore, PM2.5 may aggravate asthma via Th17/Treg imbalance, specifically by decreasing the level of IL-10 and increasing the levels of HB-EGF, IL-17 and TGF- β1. FA (19.4mg/kg, 38.8mg/kg) could antagonize the change in Th17/Treg imbalance induced by PM2.5 in asthmatic rats.

Effect of FA on morphological changes in lung tissues induced by PM2.5 in asthmatic rats

Typical pathological features of asthmatic airway inflammation and structural alterations, including leukocyte infiltration, epithelial folding, and thickened subepithelial cell layers were demonstrated after H&E staining. In the control group, there was minor inflammatory cell infiltration near the lung and bronchus, alveolar structure of bronchial

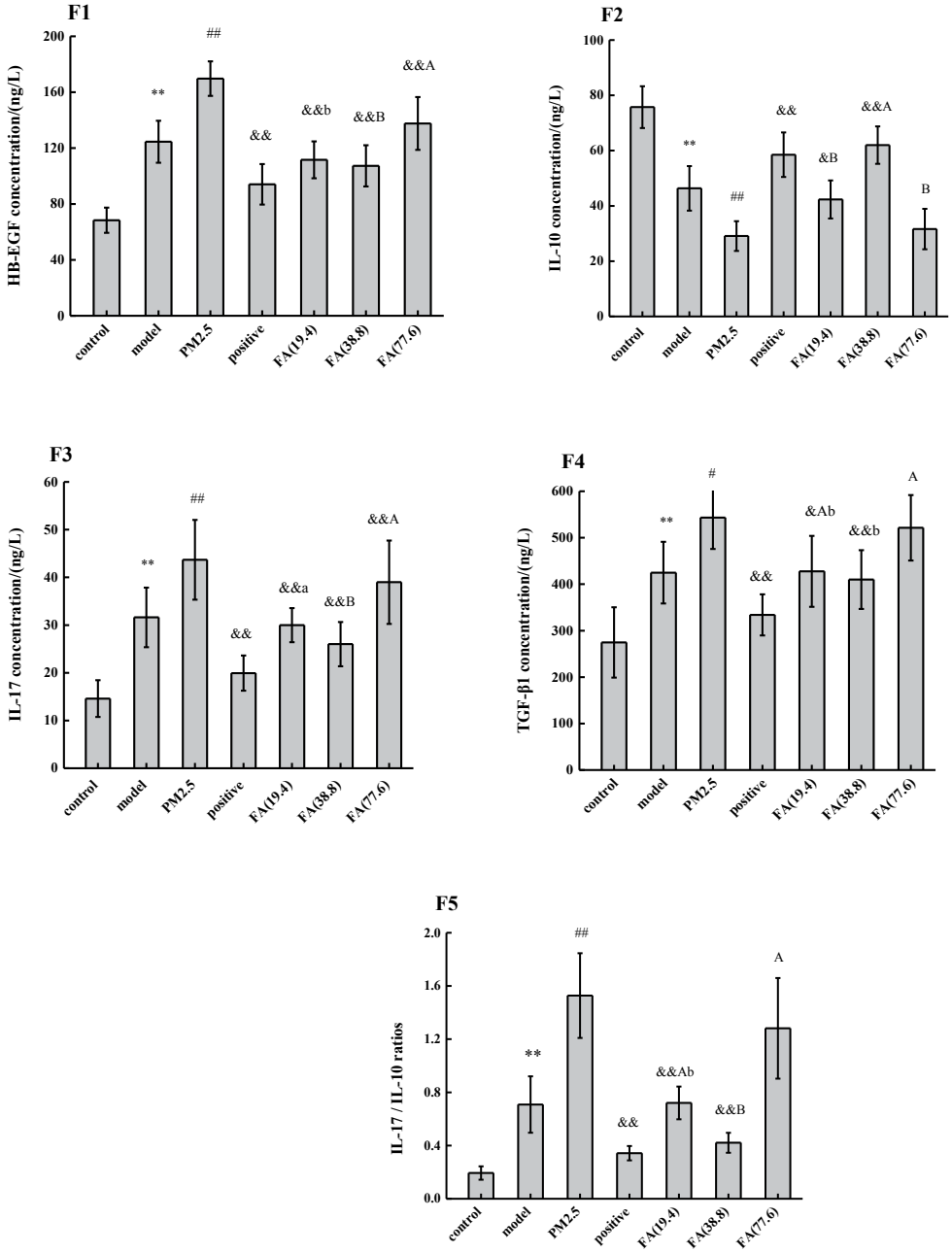


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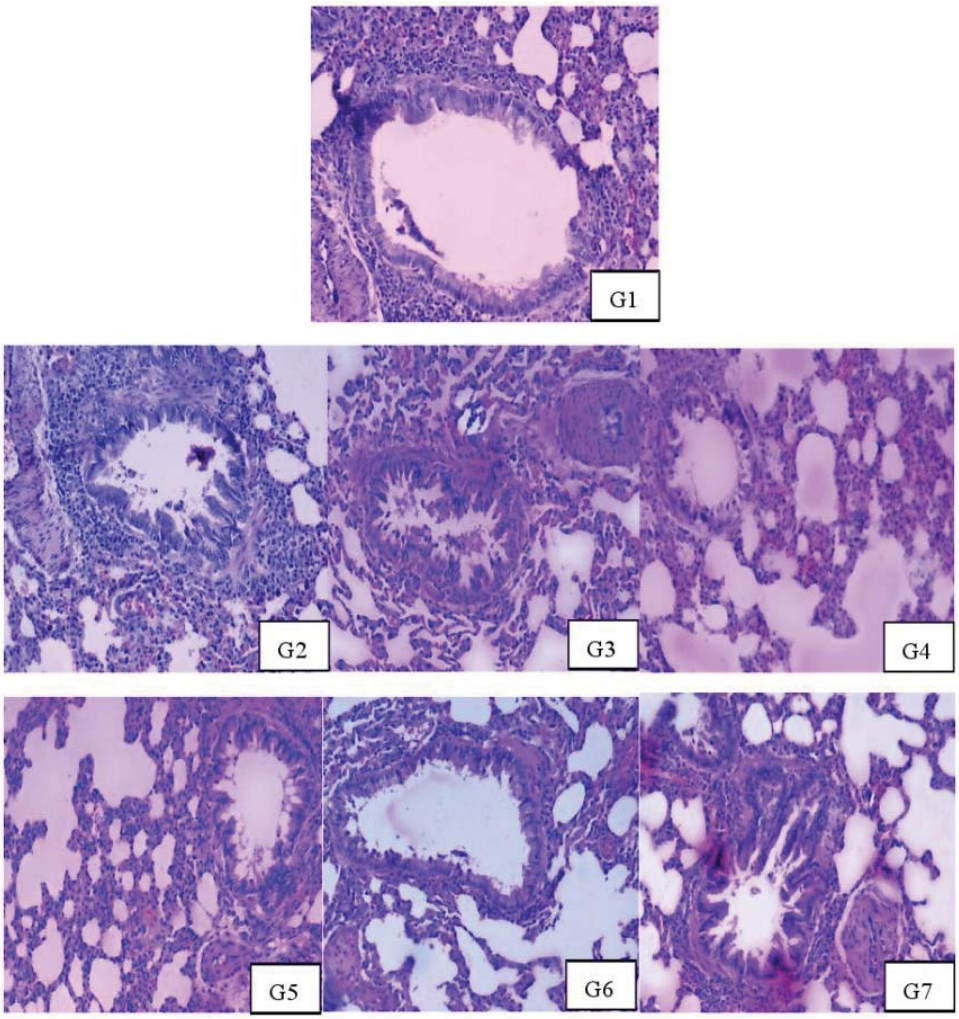
Figure 7: Effect of FA on the imbalance of Th17/Treg induced by PM2.5 in asthmatic rats.

epithelial was of normal integrity, and there was minimal exudate in the bronchial lumen (Figure 8G1). In the model group, epithelial cells were incomplete, and there were lumens stenosis, wall collapse, basement membrane thickening, substantial exudate, pulmonary interstitial thickening and inflammatory cell infiltration in different degrees of pathological changes (Figure 8G2). In the PM2.5 group, lesion degree deepened, bureaucratic narrow wall shrinkage was more remarkable, and there was alveolar septum fracture (Figure 8G3). The positive group and the FA groups had lower degrees of inflammatory cell infiltration and mucus secretion, suggesting improved bronchial injury (Figure 8G4-G7).

Discussion

Our study successfully established a rat allergic asthma model of airway inflammation. The aggravating effects of PM2.5 on OVA-induced allergic asthma were also verified by detecting serum cytokines and lung Ig concentrations, airway leukocyte infiltration and histopathological changes in lung tissues. To further study the effects of PM2.5 on asthmatic rats and the protection of FA, other serum and BALF cytokines and the factors of cytotoxicity were also detected. We found that PM2.5 (6.0mg/kg) could aggravate lung injuries in OVA-sensitized asthmatic rats by improving inflammatory cell infiltration and disturbing the balance of Th1/Th2 and Th17/Treg. FA has antagonistic effects against the respiratory tract injuries caused by PM2.5 in asthmatic rats. Treatments with FA in 19.4 mg/kg and 38.8 mg/kg had significant protective effects, and the protective mechanism may be related to affecting the balance of Th1/Th2 and Th17/Treg by reducing the total number of inflammatory cells, decreasing the levels



Note: G1~G7 stand for the following groups, respectively.

G1.control; G2.model; G3. $PM_{2.5}$; G4.positive; G5. FA(19.4 mg/kg); FA(38.8mg/kg); G7. FA(77.6 mg/kg).

Figure 8: Histopathological changes in lung tissues in various groups (× 200).

of cytokines IL-4, IL-5, TNF-α, MIP-1α, Eotaxin-1, MMP-9, TIMP-1, IL-8 in serum, TGF-β1, HB-EGF and IL-17 in BALF and antibodies IgE, OVA-sIgE in lung, and increasing the levels of IFN- γ in serum and IL-10 in BALF.

Allergic asthma is a complex and chronic inflammatory airway disease, and many cells such as eosinophils, T lymphocytes and neutrophils cells are involved in the development of the disease. The imbalances in Th1/Th2 and Th17/Treg immunity play an important role in the pathogenesis of allergic asthma [10,7]. The effect and mechanism of PM2.5 on asthma have been investigated in previous studies. PM2.5 can induce local and systemic inflammatory reactions, causing the imbalance in the Th1/Th2 pathway [20]. However, the mechanism of PM2.5-induced asthma is still unclear. At present, corticosteroids inhalation is the conventional treatment for asthma [21], but long-term usage may lead to hypertension, osteoporosis and other side effects. Therefore, investigation for more effective and safer bioactive substance has become one of hot topics in the treatment of bronchial asthma. Ferulic Acid, a natural bioactive substance, has good antioxidant and anti-inflammatory properties and protective effect against cardiovascular disease [22-24]. Our study results demonstrated that PM2.5 could aggravate airway inflammation in OVA-sensitized asthmatic rats by increasing the inflammatory cell infiltration and inducing the imbalance of Th1/Th2 and Th17/Treg, while FA could effectively antagonize these adverse effects, providing a potential universal strategy for the effective protection of people with allergic asthma from PM2.5 or similar PM-induced aggravation.

Total IgE and OVA-sIgE concentrations are regulated by the balance of Th1/Th2 and are important in the diagnosis of asthma [25,26]. Inflammatory cell recruitment in the airway is a key event in the pathological progress of asthma [27]. What must be mentioned is that eosinophils are regulated in terms of their recruitment, activation, growth, differentiation and survival by IL-5. Abundant data from *in vitro* experiments, animal models and clinical trials have highlighted that IL-5 inhibition may be an effective approach for the treatment of asthma, especially severe asthma [28]. The expression of IFN-γ (a Th1 cytokine) correlates with antigen-induced airway hyperresponsiveness and eosinophilic infiltration [29,30]. TNF-α is a pro-inflammatory cytokine that has been implicated in many aspects of the airway pathology in asthma. It is primarily produced by macrophages and is also produced by several other pro-inflammatory cells, including dendritic cells, B cells, neutrophils and eosinophils, and so on [31]. In this study, the observed changes in serum cytokines and lung Ig concentrations as well as the BALF cell profile indicated that 6.0mg/kg PM2.5 treatment on asthmatic rats further aggravated OVA-induced allergic inflammation. The exacerbating effects of PM2.5 on airway allergic inflammation have been verified in many previous papers [32-34], and results in this study are in agreement with those reported.

In order to confirm that the mechanism of the effects of PM2.5 on asthmatic rats may be associated with the imbalance of Th1/Th2 and Th17/Treg, the levels of IFN-γ, IL-4, IL-5 in serum, IL-10 and IL-17 in BALF were detected to calculate the ratios of IFN-γ to IL-4 and IL-17 to IL-10. In this study, the levels of IL-4, IL-5, IL-17, TGF-β1

(Figure 5E2-E3, Figure 6F3-F4) were increased and the levels of IFN-γ and IL-10(Figure 5E1 and Figure 6F2) were decreased in the PM2.5 group compared with the model group, together with the increases in IL-17/IL-10 ratios (Figure 4C5) and the decreases in IFN-γ/IL-4 ratios (Figure 5E5). It showed that PM2.5 can aggravate asthma by regulating the balances of Th1/Th2 and Th17/Treg. Humoral immunity is a major response in the process of respiratory system injury caused by airborne PM2.5 in rats with unbalanced Th1/Th2 ratios [10]. PM2.5 can also aggravate the Th1/Th2 and Th17/Treg immune imbalance in COPD mice of serum, as demonstrated by increases in Th1%, Th17%, Th1/Th2, Th17/Treg, INF-γ and IL-17, and decreases in Th2%, Treg%, and IL-4 and IL-10 [35]. Meanwhile, results from FA treatment also suggest that the mechanism of the effects of FA on PM2.5-induced asthmatic rats may be associated with the imbalance of Th1/Th2 and Th17/Treg. HB-EGF is a potential biomarker for the active stage of ASM remodeling [36] and the level of HB-EGF expression is correlated with the ASM mass thickening [37]. It is found that the over-expression of HB-EGF as a IL-17 downstream molecule played an indispensable role in the process of Th17-induced airway remodeling [38]. In this study, the level of HB-EGF in the model group was greater than that in the control group and in the PM2.5 group was greater than that in the model group. Different doses of FA could reduce HB-EGF concentrations to various extents [Figure 6F1].

Asthma is a chronic inflammatory disease that is mediated by a number of chemokines. To investigate the effect of FA on chemokines induced by PM2.5 in asthmatic rats, the levels of Eotaxin-1, IL-8 and MIP-1α were measured. Eotaxin-1 belongs to the CC chemokine family that attracts circulating Th2-lymphocytes and eosinophils from the blood stream to the inflammatory foci. Eotaxin contributes to the eosinophil chemotactic activity of sputum from subjects with more severe forms of asthma but not mild asthma, suggesting that its contribution is more important in more severe disease [39]. The expression of Eotaxin-1 protein and mRNA is significantly increased in the epithelium of airway, proportional to eosinophil infiltration in asthmatic patients [40]. One animal study has reported that smoke increases the levels of Eotaxin-1 expression under co-exposure to other allergens, leading to airway inflammation [41]. It has been shown that MIP-1α is a key mediator in eosinophils in lung diseases, and it can promote the migration of eosinophils [42]. IL-8 plays an important role in attracting inflammatory cells to the inflammatory region, and therefore it may serve as the main factor for airway inflammation in asthmatic area [43]. Neutrophils are the dominant inflammatory leukocyte characterizing airway inflammation in acute severe asthma that requires mechanical ventilation, and that IL-8 is an important mediator of this neutrophilia [44]. In this study, the levels of Eotaxin-1, IL-8 and MIP-1α in the model group was greater and in the PM2.5 group were even greater than that in the model group. Different doses of FA could reduce Eotaxin-1, IL-8 and MIP-1α concentrations to various extents [Figure 3C1-C3].

MMP-9 and its inhibitor TIMP-1 are involved in tissue inflammatory processes and fibrogenic processes. Previous reports have suggested that MMP-9/TIMP-1 ratio may reflect the balance between these two processes in various diseases [45]. TIMP-1 is synthesized and released by human neutrophils and can bind both the active and pro-form of MMP-9 [46]. The over-expression of MMP-9 can not only result in ECM remodeling, but also can induce the migration of eosinophils and airway smooth muscle cell proliferation and migration induced by asthma airway remodeling [47,48]. In this study, the levels of MMP-9 and TIMP-1 in the model group was higher than those in the control group and in the PM2.5 group were greater than those in the model group. Different doses of FA could decrease MMP-9 and TIMP-1 concentrations to various extents [Figure 4D1 and 4D2]. However, there was no significant change in the ratio of MMP-9 /TIMP-1 in all groups. Furthermore, the existing literature suggests that PM2.5 exposure can induce cytotoxicity in asthmatic rats by regulating the activities of ACP, AKP and LDH [49,50]. The activities of ACP, AKP and LDH in the model group was greater than those in the control group and in the PM2.5 group were greater than those in the model group. Different doses of FA could decrease ACP, AKP and LDH activities to various extents [Figure 1A1-A3].

The researches on FA are always focused on antioxidant and anti-inflammatory effects. FA has good antioxidant activity that can participate in a variety of oxidation-reduction of Fe^{2+} combination, reducing oxidation and reduction reactions [51], can clear superoxide radicals, hydrogen peroxide, hydroxyl radical and Nitro oxide, also can inhibit the body's enzymes to produce free radicals, protecting the body from oxidative damage [52,53]. The pre-treatment of FA could prevent the increased mitochondrial ROS, apoptosis rate and expression of caspase-3 and caspase-9, and the decreased mitochondrial membrane potential, mPTP activity, and expression of PGC-1α and Mn-SOD of A549 caused by PM2.5 [54]. PM2.5 causes damage on CHO cells through a variety of mechanisms ,and FA can attenuated these effects by reducing oxidative stress and the over-up-regulatedinduction of Akt and NF-KB pathways [55]. FA has a neurogenic inflammation inhibitory effect by extracellular signal-regulated kinase (ERK) signaling pathway [18] ,and its anti-inflammatory effect is mainly manifested in inhibiting cytokines expression, such as IL-6, TNF-α, and activating metallothionein, such as MT-1, MT-2 [56]. FA has exciting resistance of poisoning and antidepressant-like effects [57]. Analgesic effect may be related to inhibition of monoamine oxidase activity in rat brain, thereby increasing the amine neurotransmitters in the brain-related [58]. FA can reduce the levels of OVA-specific immunoglobulin E (IgE) and IgG1 and enhanced IgG 2a antibody production in serum and inhibit the production of eotaxin, Th2 cytokines (IL-4, IL-5, and IL-13), and pro-inflammatory cytokines but promote the production of Th1 cytokine(IFN-γ)in bronchoalveolar lavage fluid (BALF) and the culture supernatant of spleen cells [59]. Therefore, the abundant dietary sources, the relatively low toxicity, and potential bioactivities of FA may be capable of influencing immune cell functions in allergic immune responses and provide possible alternative options for relieving allergic asthma-associated symptoms.

In our study, FA in 19.4mg/kg, 38.8 mg/kg and 77. 6 mg/kg were pre-treated for PM2.5-induced asthmatic rats. FA in 19.4 mg/kg and 38.8 mg/kg had significant protective effects against the adverse effects induced by PM2.5, but FA in 77. 6 mg/kg had no significant protective effects. It might be related to 77. 6 mg/kg FA that were not within the effective dose. FA is a phenolic acid of low toxicity [60]. FA is most likely to distribute in kidney in rats, followed by the lungs, liver, spleen, heart, uterus, and FA excretion in rats is mainly through urinary [61]. FA-induced metabolic responses in animals have only a few reports until now [62]. Furthermore, the strong antioxidative activity of FA is the result of a variety of mechanisms [63]. To date, the protective effects of FA against chronic inflammatory lung diseases, such as asthma, and the underlying mechanisms are still uncertain. In our study, FA successfully alleviated lung inflammation and reduced infiltration of total inflammatory cells in peribronchiolar regions. Based on these findings, we speculate that FA inhibits the recruitment of inflammatory cells by regulating the balance of Th1/Th2 and Th17/Treg and related cytokines ,chemokines providing a possible mechanistic explanation for the observed profound regulatory effects of FA on the development of airway inflammation in PM2.5-induced asthma rats.

Conclusion

In conclusion, the current study demonstrated the ability of PM2.5 to aggravate asthma and the ability of FA to protect the respiratory injuries in PM2.5-induced asthmatic rats. FA (19.4, 38.8 mg/kg) had significant protective effects against the adverse effects induced by PM2.5. The mechanism for FA protection may be related to the regulation of balances between Th1/Th2 and Th17/Treg, as demonstrated by decreases in total number of inflammatory cells and

the levels of cytokines (IL-4, IL-5, TNF-α, MIP-1α, Eotaxin-1, MMP-9, TIMP-1, IL-8, TGF-β1, HB-EGF and IL-17) and antibodies (IgE, OVA-sIgE), and increases in the levels of cytokines IFN-γ and IL-10. These changes could inhibit the airway inflammatory response induced by PM2.5 in asthmatic rats effectively.

Conflict of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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