

The effect and mechanism of Modified Guo-Min Decoction and Yu-Ping-Feng Powder on fine particulate matter-induced lung inflammatory injury and airway mucus hypersecretion in rats

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Research

Keywords: Fine particulate matter (PM2.5), Lung inflammatory injury, Mucus hypersecretion, Modified Guo-Min Decoction (MGMD), Yu-Ping-Feng Powder (YPPF)

Posted Date: May 7th, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-26075/v1>

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Abstract

Background

Exposure to fine particulate matter (PM_{2.5}) severely impairs public health. The mechanism of PM_{2.5}-induced lung injury is complex and diverse. Modified Guo-Min Decoction (MGMD) and Yu-Ping-Feng Powder (YFPF) have been found to improve clinical symptoms in respiratory patients during smog weather, but the mechanism remains unclear. This study aimed to investigate the effect and mechanism of YFPF and MGMD against PM_{2.5}-induced lung injury.

Methods

We established the PM_{2.5} animal model by intratracheal instilling of PM_{2.5} suspensions. Rats were administrated MGMD/YFPF/distilled water via gavage every day, and all rats were sacrificed after 28 days. At the end of experiment, BALF and lung tissues were collected. Condition of lung injury, inflammatory cells infiltration, inflammatory cytokines, MUC5AC synthesis and release, and phosphorylation of TLR2-MyD88-NFκB and EGFR-PI3K-AKT signalling pathway were evaluated.

Results

The results demonstrated that both MGMD and YFPF protected rats from PM_{2.5}-induced damaged structure of lung tissues. The infiltration of neutrophil, monocyte, lymphocyte, and eosinophil was reduced after the treatment of two therapies. The production of pro-inflammatory mediators, MCP-1 and NE, as well as the type2 inflammation-related cytokines, IgE and IL-4, were decreased by MGMD and YFPF. However, the MGMD showed more potent effect on inhibiting IL-4, while YFPF benefited in preventing ICMA-1, IL-1β, and IL-17A. Rare significance was detected in the TLR2-MyD88-NFκB of each group. Treatment with MGMD and YFPF decreased goblet cell hyperplasia and the expressions of MUC5AC. The further investigation demonstrated that YFPF had the effect of simultaneously inhibiting the phosphorylation of PI3K and AKT, whereas MGMD only showed a significant difference in AKT.

Conclusions

Therefore, both MGMD and YFPF could significantly attenuate PM_{2.5}-induced inflammation of lung and airway mucus hypersecretion. Nevertheless, YFPF had more advantage in preventing type1 inflammation and mucus hypersecretion, while MGMD was more beneficial in reducing type2 inflammation.

Background

According to the latest World Health Statistics published in 2019, around 91% population of the world lived in the circumstance where the air quality inferior to WHO's suggestion, and an estimated 4.2 million

people died due to ambient air pollution. Fine particulate matter (PM_{2.5}) which less than 2.5 microns in diameter is the major source of air pollutant[1]. The average annual concentration of PM_{2.5} in Chinese urban area in 2016 (51 µg/m³) was far beyond the level WHO recommended (12 µg/m³). Recent epidemiological studies have shown that both long and short term exposure to PM_{2.5} are closely associated with respiratory diseases which increased the risk of chronic obstructive pulmonary disease (COPD), lung cancer, asthma and pneumonia[2–4]. The mechanisms of PM_{2.5}-induced respiratory system injury have been investigated including inflammatory response, oxidative stress, genotoxicity, and imbalanced intracellular calcium homeostasis[5, 6].

The inflammatory response which runs through the process of several respiratory diseases is the major cause of PM_{2.5}-induced lung injury. PM_{2.5} destroys the epithelium and initiates the inflammatory response by stimulating epithelium cells and pulmonary macrophage to express cytokines, e.g., the interleukin (IL)-1 β , interleukin (IL)-6, interleukin (IL)-17A, tumor necrosis factor (TNF)- α , and Monocyte chemoattractant protein (MCP)-1[7]. These inflammatory factors can activate inflammatory cells infiltration and attraction such as neutrophils, mononuclear macrophages, and lymphocytes which will aggravate inflammatory injury and immune function in turn[8]. The TLR2-MyD88-NF κ B signalling pathway is closely associated with inflammatory response and plays a vital role in multiple respiratory diseases [9]. Toll-like receptor (TLR) 2 is a transmembrane protein which is a critical component of detecting pathogen and initiating immunity in the respiratory epithelium[10, 11]. After activated by inflammasomes, TLR2 induces the recruitment of myeloid differentiation primary response gene 88 (MyD88) by ligand binding. Consequently, the downstream nuclear factor kappa B (NF κ B) is phosphorylated. Typical NF κ B structure is the heterodimer consisting of two subunits, RelA (p65) and p50. After activated, NF κ B translocates into the nucleus, leading to the expression of inflammatory mediators[12]. NF κ B is not only a part of inflammatory processes but an important role in tumorigenic function. The main target genes of TLR2-MyD88-NF κ B signalling pathway include IL-1, IL-6, IL-8, TNF- α , MCP-1, vascular cell adhesion molecule 1 (VCAM-1) and the intercellular cell adhesion molecule 1 (ICAM-1). Recent studies showed that PM_{2.5} could lead to exacerbation of airway inflammation by activating TLR2-NF κ B signalling pathway [13]. Type 2 inflammation which involves eosinophils, mast cells, basophils, T_H2 cells is another important mechanism of respiratory diseases, such as asthma, bronchial hyperresponsiveness, and allergic rhinitis. These immune cells secrete type 2 cytokines (e.g., IL4, IL5 and IL13), IgE, and bioactive substance which is essential for airway remodeling and mucus hypersecretion. [14]. A study from Japan discovered that exposure to PM_{2.5} could cause severe eosinophil infiltration in airways and increase the level of IL-4, IL-5, and IL-13 in bronchoalveolar lavage fluid (BALF) of BALB/c mice[15].

Mucus hypersecretion is both the pathogenic factor and pathological product of various airway diseases including asthma, chronic obstructive pulmonary disease, and cystic fibrosis. Airway mucus which major component is mucin5AC (MUC5AC) is mainly secreted by goblet cells in the bronchial epithelium[16]. It has been investigated that PM_{2.5} could induce MUC5AC expression in the rat airway after 3 months' exposure[17]. MUC5AC synthesis and release can be initiated by several signalling pathway especially

EGFR-PI3K-AKT. Stimuli such as PM2.5 and inflammatory mediator activate epidermal growth factor receptor (EGFR) across the cell membrane. These transduction signals motivate the downstream phosphatidylinositol- 4,5-bisphosphate 3-kinase (PI3K) and protein kinase B (AKT) to phosphorylate which induces MUC5AC mRNA expression in nuclear[18]. A research in vitro showed that PM2.5 induced mucus hypersecretion by activating EGFR-AKT pathway in human bronchial epithelial cell (HBEC)[19].

Modified Guo-Min Decoction (MGMD) is transformed from Guo-Min decoction (GMD) which is an empirical formula designed by TCM physician Zhu Chenyu. GMD is consist of Radix Stellariae (Yinchaihu), Radix Saposhnikoviae (Fangfeng), Fructus Mume (Wumei), and Fructus Schisandrae Chinensis (Wuweizi). This formula is mainly applied in treatments for allergic diseases such as asthma, cough variant asthma (CVA), allergic rhinitis, and atopic dermatitis. The mechanism might be associated with downregulating IL-4 and IL-5 mRNA expression in lung tissues[20]. In clinical therapy, we found that GMD with the addition of Radix Peucedani (Qianhu) and Radix Platycodonis (Jiegeng) was effective in patients with PM2.5-induced cough and phlegm.

Yu-Ping-Feng Powder (YPPF) which consist of Radix Astragali (Huangqi), Radix Saposhnikoviae (Fangfeng), and Rhizoma Atractylodis Macrocephalae (Baizhu) is a classic Chinese medicine formula recorded in *Jiu Yuan Fang*. YPPF was widely used in respiratory diseases for the main mechanism that it could relieve inflammation and promote transformation of immunological cells[21, 22]. In the clinical application, we found that YPPF could alleviate PM2.5-induced respiratory symptoms. However, the underlying mechanisms of both two formulas on PM2.5-related pulmonary injury remain unclear. In this study, we investigated and compared the effects of MGMD and YPPF on PM2.5-induced lung injury of Wistar rat and their mechanisms.

Materials And Methods

Medication preparation

MGMD is composed of Radix Stellariae (Yinchaihu) 10 g, Radix Saposhnikoviae (Fangfeng) 10 g, Fructus Mume (Wumei) 10 g, Fructus Schisandrae Chinensis (Wuweizi) 10 g, Radix Peucedani (Qianhu) 10 g, Radix Platycodonis (Jiegeng) 10 g. YPPF consists of Radix Astragali (Huangqi) 30 g, Radix Saposhnikoviae (Fangfeng) 10 g, and Rhizoma Atractylodis Macrocephalae (Baizhu) 10 g. Both MGMD and YPPF were made into granules by Beijing Kangrentang Pharmaceutical Co., Ltd. (Beijing, China). The granules were then suspended in distilled water to a final concentration of 0.189 g/mL and 0.21 g/mL respectively and the liquid was stored at 4°C.

PM2.5 suspension preparation

The PM2.5 collecting site was on the roof of Chinese Research Academy of Environmental Sciences (Beijing, China). The sample was collected on Teflon filters (Pall Corporation, New York, USA) 8 h/day for consecutive 2 weeks in January 2018 by using Thermo Anderson sampler (PDR-1500, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The filter membrane was then cut into 2 × 2 cm sections and

immersed in distilled water for ultrasonic oscillation at room temperature for 30 min x 3 times. After filtrated with gauze, the solution was dried overnight in a freezing vacuum machine (LABCONCO, Kansas City, MO, USA). The particles were weighed and resuspended in normal saline (NS) to a final concentration 0.65 g/mL.

Animal and experimental protocol

Twenty Wistar rats (male, 6–8 weeks old, weighing 180–220 g) were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). All rats were kept in a 12 h dark/light cycle and constant temperature (25°C) room with enough food and sterile water. The experimental protocol was approved by the Experimental Animal Ethics Committees of Peking University People's Hospital (No. 2017PHC022).

The rats were randomly separated into four groups which were Control group (n = 5), PM2.5 group (n = 5), MGMD group (n = 5) and YFPF group (n = 5). Every three days from the first day of the experiment, PM2.5 group, MGMD group and YFPF group were intratracheal instilled 0.1 mL PM2.5 suspensions via rats' throat using a 18G remaining catheter after anesthesia with 5% isoflurane, while Control group was instilled 0.1 mL NS.

Rats in Control and PM2.5 groups were intragastrically administrated distilled water (10 mL/kg·bw); MGMD group was administrated MGMD solution (10 mL/kg·bw) via gavage; YFPF group was given YFPF solution (10 mL/kg·bw). All treatments were administrated once per day for 28 days (Fig. 1).

Sample harvesting

On day 29, after anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg·bw) all rats were sacrificed by blood collection from abdominal aorta. Opened rat's chest, tied up right lung hilum and isolated right lung. The middle lobe was fixed by 4% paraformaldehyde for 48 h, dehydrated in a gradient ethanol series, and embedded in paraffin for morphology detection. The anterior and posterior lobes of right lung were stored in liquid nitrogen for qPCR and Western Blot analysis. 5 mL PBS infused into right lung and pumped 3 times to collect bronchoalveolar lavage fluid (BALF). The liquid was then centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was aspirated and maintained at -80°C for ELISA analysis.

HE and AB/PAS-Staining

The paraffin blocks were cut into 4 µm sections. The sections were sequentially deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E). After dehydrated and mounted, the sections were finally observed under light microscope (Leica DFC400, Germany).

Alcian Blue/Periodic Acid-Schiff (AB/PAS) staining was performed to detect goblet cell metaplasia of bronchial epithelium. The images of lung tissues were captured by microscope. AB/PAS positive area and total area of corresponding bronchial epithelium were measured by the software Image-Pro Plus 6.0. Data were presented as the ratio of AB/PAS-positive area to the total area.

ELISA assay

The levels of inflammatory cytokines in BALF were measured by using rat IL-1 β ELISA kit (NOVUS, USA), rat IL-6 ELISA kit (NOVUS, USA), rat TNF- α ELISA kit (NOVUS, USA), rat IgE ELISA kit (NOVUS, USA), rat ELISA IL-4 kit (NOVUS, USA), rat IL-5 ELISA kit (Signosis, USA) and rat IL-13 ELISA kit (abcam, USA) according to the manufacturers' instructions respectively. The absorbance was measured at a wavelength of 450 nm with microplate reader (Bio-Rad, USA).

Immunohistochemistry assay

Tissues paraffin was sliced into 3 μ m sections. After being heated in 1 mM EDTA at 95°C for 20 min, the sections were incubated with 3% hydrogen peroxide for 20 min and blocked by 10% goat serum for 90 min at room temperature. Section was then incubated overnight at 4°C with 100 μ L of rabbit polyclonal antibody against MPO (abcam, USA, dilution 1:50), 100 μ L of mouse monoclonal antibody against CD68 (abcam, USA, dilution 1:200), 100 μ L of rabbit polyclonal antibody against CD4 (proteintech, China, dilution 1:100), 100 μ L of mouse monoclonal antibody against CD8 (abcam, USA, dilution 1:200), 100 μ L of rabbit monoclonal antibody against ICAM-1 (proteintech, China, dilution 1:200), 100 μ L of mouse monoclonal antibody against MCP-1 (proteintech, China, dilution 1:200), 100 μ L of mouse monoclonal antibody against MUC5AC (abcam, USA, dilution 1:500), 100 μ L of rabbit polyclonal antibody against NE (abcam, USA, dilution 1:2000). After being washed in 1 \times PBS, the sections were incubated with HRP-conjugated goat anti-rabbit or anti-mouse second antibody (zsbio, China, dilution 1:400) for 1 h at room temperature. Then sections were color developed with 3,3-diaminobenzidine tetrahydrochloride (DAB). The yellow brown stains were positive for target proteins. The images were captured by light microscope (Leica DFC400, Germany).

Real-Time qPCR

Total RNA was extracted from anterior and posterior lobes of right lung by using Trizol reagent (Thermo scientific, USA), chloroform and isopropanol following the classic protocol. RNA reverse transcription was proceeded by using RevertAid First Strand cDNA Synthesis Kit (Thermo scientific, USA) referring to the manual. The real-time PCR reactions were performed by using Power SYBR[®] Green PCR Master Mix (Thermo scientific, USA). The thermal cycling parameters included 95°C for 10 min to activate and 40 cycles of 95°C for 15 s and 60°C for 1 min. The primers of MUC5AC and reference gene GAPDH were synthesized by Invitrogen (Beijing, China). The primers sequences are listed below: MUC5AC: Forward 5'-TCCTTCTCCACAACGCTGGTCA-3', Reverse 5'-ATACGCTGCTGGCTGTCAACAC-3'; GAPDH: Forward 5'-GGCAAGTTCAACGGCACAGTCA-3', Reverse 5'-ACGCCAGTAGACTCCACGACAT-3'. $2^{-\Delta\Delta CT}$ represented the relative expression of genes.

Western Blot analysis

20 mg lung tissues were lysed in 200 μ l mixture of RIPA (Beyotime, China) and PMFS (Beyotime, China). After being centrifuged (4°C, 14000 g for 5 min), total proteins were extracted. The protein concentration was determined by BSA kit (keygentec, China) and diluted to final concentration 3 μ g/ μ L with distilled

water and 4 × protein loading buffer. 10µL protein mixture was separated by 6%, 8% or 10% SDS-PAGE gel electrophoresis (Beyotime, China), and transferred to PVDF membranes (Pall Corporation, USA). After being blocked with 5% nonfat dry milk (BD biosciences, USA) in 1 × TBST at room temperature for 1 h, the membranes were incubated with primary antibodies diluted in 5% nonfat dry milk as following: rabbit monoclonal antibody against MPO (abcam, USA, dilution 1:1000), rabbit polyclonal antibody against CD68 (abcam, USA, dilution 1:1000), rabbit polyclonal antibody against CD4 (proteintech, China, dilution 1:500), mouse monoclonal antibody against CD8 (abcam, USA, dilution 1:250), mouse monoclonal antibody against MCP-1 (proteintech, China, dilution 1:2000), rabbit monoclonal antibody against ICAM-1 (abcam, USA, dilution 1:5000), rabbit polyclonal antibody against TLR2 (affinity, China, dilution 1:500), rabbit polyclonal antibody against MyD88 (affinity, China, dilution 1:500), rabbit monoclonal antibody against NFκB p65 (abcam, USA, dilution 1:2000), rabbit polyclonal antibody against phospho-NFκB p65 (abcam, USA, dilution 1:2000), rabbit polyclonal antibody against MUC5AC (Santa Cruz, USA, dilution 1:200), rabbit polyclonal antibody against NE (abcam, USA, dilution 1:500), rabbit polyclonal antibody against EGFR (proteintech, China, dilution 1:2000), rabbit polyclonal antibody against phospho-EGFR (CST, USA, dilution 1:2000), rabbit monoclonal antibody against PI3K (abcam, USA, dilution 1:1000), rabbit polyclonal antibody against Phospho-PI3K (abcam, USA, dilution 1:1000), rabbit polyclonal antibody against AKT (CST, USA, dilution 1:1000), rabbit polyclonal antibody against phospho-AKT (CST, USA, dilution 1:1000), and mouse monoclonal antibody against β-actin (abcam, USA, dilution 1:5000) served as internal control at 4°C overnights. After being washed with 1 × TBST, the membranes were incubated with HRP-conjugated second antibody (proteintech, China, dilution 1:5000) at room temperature for 1 h. At last, the bands were captured by gel-imaging system (GE Healthcare, USA) with ECL reagent (Forscience, China) and measured by Image J software (NIH, MD, USA).

Statistical analysis

The result was expressed as mean ± SD. We use SPSS 22.0 software (IBM; Armonk, NY, USA) to determine the statistical differences between groups by One-way analysis of variance (ANOVA). $P < 0.05$ was set as statistically significant.

Results

Effect of MGMD and YFPF on PM2.5-induced Pulmonary Histopathological Injury

In Control group, the airway epithelial cells with intact cilia arranged regularly. In lamina propria of normal rats, there were few inflammatory cells. It could be hardly found hyperplastic gland around the airway. In the visual field of alveoli, the structure remained integrity without alveolar fusion, collapse, interstitial edema, or hyperemia. Compared with Control group, lung of PM2.5 group showed obvious histological injuries such as thickened and swollen epithelium, adhesion and loss of cilia, gland hyperplasia, alveolar fusion and collapse, interstitial edema and congestion, and inflammatory cells infiltration. In both MGMD and YFPF groups, the epithelial injures and alveoli destruction were significantly inhibited compared with

PM2.5 group (Fig. 1A). As shown in Fig. 1B, airway smooth muscle in PM2.5 group and YFPF group has the hypertrophy trend compared with Control group while the area of airway smooth muscle in MGMD group tended less than that in PM2.5 group. However, there was no statistical significance between each group. In terms of eosinophils around the airway, there was a remarkable increase in PM2.5 group compared to those in Control group (0.93 ± 0.55 vs 9.00 ± 0.85 , $P < 0.01$). Mean counts of eosinophils in both MGMD and YFPF groups have noticeable decrease compared with PM2.5 group (4.07 ± 2.70 vs 9.00 ± 0.85 , $P < 0.01$ and 4.00 ± 0.78 vs 9.00 ± 0.85 , $P < 0.01$, Fig. 1C).

Effect of MGMD and YFPF on PM2.5-induced Inflammatory Cells Infiltration and Mediators Expression

The effects of MGMD and YFPF on the lung of PM2.5 exposed rats were determined by molecular biology experiments on the inflammatory cells surrounding the airway, cytokines in BALF, and inflammatory mediators in lung tissues. We observed that prolonged exposure to PM2.5 could increase the infiltration of the inflammatory cell around the airway. MPO, CD68, CD4, and CD8 were the specific markers of activated neutrophil, macrophage, and T lymphocytes, respectively. As shown in Fig. 2, compared with Control group, multiple inflammatory cells in the submucosa and lamina propria of the airway were significantly increased in PM2.5 group. After being intervened by MGMD and YFPF, the infiltration was restrained to a certain extent.

The level of cytokines in BALF reflected the inflammatory conditions of the lungs. In this study, we measured IL-1 β , IL-6, TNF- α , IL-17A, IgE, IL-4, IL-5, and IL-13 by ELISA assay. As shown in Fig. 3, exposed to PM2.5 could remarkably increase the level of IL-1 β ($P < 0.01$), IL-17A ($P < 0.05$), IgE ($P < 0.01$), and IL-4 ($P < 0.01$) with statistic difference. Compared with PM2.5 group, the levels of TNF- α ($P < 0.05$), IgE ($P < 0.01$), and IL-4 ($P < 0.01$) in MGMD group and IL-1 β ($P < 0.01$), IL-17A ($P < 0.05$), IgE ($P < 0.01$), and IL-4 ($P < 0.05$) in YFPF group were significantly decreased. There was no statistical significance comparing the level of IL-6, IL-5, and IL-13 in 4 groups ($P > 0.05$)

ICAM-1 and MCP-1, the inflammatory mediators, are expressed by airway epithelial cells after being stimulated by the pathogen. These mediators could recruit neutrophil and macrophage and induce inflammatory responses. Neutrophil elastase (NE), another symbol of inflammation, is released by activated neutrophils. As illustrated in Fig. 4A-C, prolonged exposure to PM2.5 could significantly increase ICAM-1, MCP-1, and NE secretion for massive positive staining was observed in PM2.5 group. We detected that treating with compounds could alleviate the mediators' synthesis in epithelia. We found the statistical significance of inhibitory effects of MGMD on MCP-1 ($P < 0.01$) and YFPF on ICAM-1 ($P < 0.01$), MCP-1 ($P < 0.01$), and NE ($P < 0.01$) via quantitative image analysis (Fig. 4D-F). The results of Western Blot analysis also manifested that the levels of ICAM-1, MCP-1, and NE in the lung tissues of PM2.5 group had significant increases compared to these of Control group. Meanwhile, the bands also showed that both MGMD and YFPF could tremendously downregulate MCP-1 and NE. Besides, the anti-inflammatory effect of YFPF was more potent for the less ICMA-1 protein expression compared with PM2.5 group (Fig. 5A-D).

Furthermore, we detected FcεRI which played an essential role in IgE releases in the lungs of each group. The data delivered that the level of FcεRI in lung tended upwards after exposure to PM2.5 with no significance. MGMD and YFPF had the inhibitory effect on FcεRI expression to a certain degree ($P < 0.01$ and $P < 0.01$) (Fig. 5A and E).

Effect of MGMD and YFPF on PM2.5-induced goblet cell metaplasia and mucus hypersecretion

Goblet cells in airway epithelium were stained bluish-violet by AB/PAS-staining. The images were captured under the light microscopy and the count of goblet cells were obtained by IPP6.0. The quantitation of goblet cell metaplasia was expressed as the ratio of positive staining area to epithelium area. As shown in Fig. 6A, it was hardly found goblet cell in Control group whereas marked hyperplasia was in PM2.5 group. Goblet cell metaplasia could also be found in MGMD group and YFPF group, but exhibited decreasing trend compared to PM2.5 group. Besides, YFPF showed more effective against goblet cell hyperplasia than MGMD. The proportion of goblet cell to epithelium were $0.35 \pm 0.36\%$, $25.56 \pm 2.58\%$, $18.17 \pm 3.45\%$ and $12.72 \pm 4.06\%$ in Control group, PM2.5 group, MGMD group, and YFPF group, respectively. There was a noticeable increase of average area of positive stains in PM2.5 group compared with Control group ($P < 0.01$). Both MGMD and YFPF could inhibit goblet cell metaplasia ($P < 0.01$, $P < 0.01$), while YFPF was more potent ($P < 0.05$) (Fig. 6B).

MUC5AC, the main component of mucus in airway, was detected by IHC staining, Western Blot and qPCR respectively in each group. As displayed in Fig. 7A, the IHC positive staining of MUC5AC was the tawny area among epithelium. MUC5AC could be rarely detected in normal epithelial lamina, while PM2.5 group resulted in significant increase. Compared with the model group, MGMD and YFPF could inhibit MUC5AC expression. The quantitation presented as the percentage of staining area among airway epithelium proved the statistical significance as the images showed. But only YFPF group manifested statistical significance (Fig. 7B). qPCR for MUC5AC mRNA presented the similar results (Fig. 7C). MUC5AC protein expression in lung tissues analyzed by Western Blot analysis indicated that PM2.5 exposures could lead to MUC5AC overexpression. Two intervention groups showed the inhibitory effects with obvious significance (Fig. 7D and E).

Effect of MGMD and YFPF on TLR2-MyD88-NFκB and EGFR-PI3K-AKT Signalling Pathway

To further investigate the mechanism of the inhibitory effects of MGMD and YFPF on inflammation and mucus hypersecretion, we applied the Western Blot analysis of the lung tissues in each group. As displayed in Fig. 8A-D, the level of TLR2 and MyD88 proteins and NFκB phosphorylation remained the same in four groups. In terms of the EGFR-PI3K-AKT signalling pathway, results showed that there were remarkable increases in phospho-PI3K ($P < 0.01$) and phospho-AKT ($P < 0.05$) of PM2.5 group compared with Control group. The phosphorylation of PI3K ($P < 0.05$) and AKT ($P < 0.05$) was decreased to varying

degrees in YFPF group with statistical difference, while MGMD only showed effective on phospho-AKT ($P < 0.05$) (Fig. 8E-G).

Discussion

According to recent clinical studies, both long and short period exposure to PM_{2.5} could increase the prevalence and mortality of respiratory diseases[2–4]. In our clinical application, we discovered that MGMD and YFPF could relieve the respiratory symptoms caused by hazy weather. However, the mechanism is still unclear. We designed this research to explore the effect and underlying mechanism of MGMD and YFPF against the PM_{2.5}-induced lung injury. We established an animal model of PM_{2.5}-induced lung injury by intratracheal instillation of PM_{2.5} suspensions and treated with MGMD and YFPF. The results of HE staining demonstrated that PM_{2.5} could induce the damage of airway epithelium, destruction of pulmonary alveoli, and inflammatory cell infiltration in lung tissues of rats. After treatment with MGMD or YFPF, we found that lung injury could be attenuated to a certain extent.

To further investigate the types of inflammatory cells, we applied immunohistochemistry and discovered that PM_{2.5} significantly increased neutrophil, macrophage, and T lymphocyte infiltration surrounding the airway, which the specific markers MPO, CD68, CD4, and CD8 were stained positive respectively. The inflammation can be staged into acute inflammation, subacute inflammation, and chronic inflammation[23]. Acute inflammation is characterized by neutrophils and monocyte infiltration, which initiated by rolling along and sticking to the epithelium. After been stimulated by PM_{2.5}, the airway epithelial cells and alveolar macrophages secreted adhesion molecule, chemokines, and cytokines. In this study, we detected that PM_{2.5} could dramatically increase ICAM-1 expression, especially in airway epithelium by morphology. We achieved the same result in the protein level of total lung tissues. ICAM-1 can lead to leukocyte adhesion and migration into airway submucosa and alveolar interstitium by binding to the ligands (e.g., LFA-1, Mac-1, integrins, and fibrinogen). Moreover, the overexpression of ICAM-1 may increase the risk of common colds, for it is also the binding site for rhinovirus[24]. MCP-1, primarily expressed by macrophages and epithelial cells is another inflammatory mediator recruiting monocytes. In the early stage of exposure to PM_{2.5}, MCP-1 elevated in the lung tissue and BALF[25]. The current study demonstrated that PM_{2.5} upregulated the MCP-1 expression in the airway epithelium and lung tissues. The same trends of IL-1, TNF- α , and IL-17A, the typical pro-inflammatory cytokines, could be found in BALF. The overexpression of NE in the lung tissues of model rats also indicated the augment and activation of neutrophils. This evidence demonstrated that PM_{2.5} could initiate acute inflammation. Except for the acute inflammatory cells, we also detected macrophages and lymphocytes infiltration and gland proliferation the characteristic in chronic inflammation. Therefore, PM_{2.5}-induced inflammatory response was the result of the combination of acute and chronic inflammation.

Both MGMD and YFPF could reduce neutrophil, monocyte, and lymphocytes infiltration for the expression of MPO, CD68, CD4, and CD8 were obviously inhibited. In terms of pro-inflammatory mediators, the results of IHC and Western Blot demonstrated that MGMD and YFPF effectively downregulated the expression of MCP-1 in lung tissues. In the study of ICAM-1, another inflammatory medium, YFPF

remarkably reduced the over-expression of ICAM-1 stimulated by PM2.5, but no significant inhibitory effect was found in MGMD group. Furthermore, the levels of IL-1 β and IL-17A in BALF and NE in lung tissue showed that YFPF was more potent in inhibiting lung tissue inflammation than MGMD.

In addition to type 1 inflammation, HE-staining showed that there was a mass of eosinophils in model rats' lung tissues, which was the symbol of type 2 inflammation. Furthermore, IgE and IL-4 closely related to type 2 inflammation exhibited higher levels in BALF after treated with PM2.5 suspensions. These mediators are essential for airway remodeling and abnormal mucus secretion. Previous evidence indicated that PM2.5 exacerbated accumulation of eosinophils and neutrophils into the airway submucosa[26, 27]. Another study reached the same conclusion that PM2.5 caused significant increase of eosinophils and neutrophils in BALF over time[28]. Therefore, we believed the PM2.5-induced inflammatory injury was the combined action of type 1 and type 2 inflammation. It could be found that both MGMD and YFPF had the inhibitory effects on PM2.5-induced eosinophil proliferation. The results indicated that MGMD and YFPF effectively decreased the levels of IL-4 and IgE in BALF, and the effect of MGMD on IL-4 was more potent. It was well acknowledged that the high affinity Fc receptor (Fc ϵ RI) played a mediating role in allergic inflammation. Fc ϵ RI mainly expressed on mast cell could bind to IgE and activate the release of histamine, prostaglandins, and leukotrienes which was known to exacerbate allergic response[29]. We detected that Fc ϵ RI protein in lung decreased markedly compared with model rats after MGMD and YFPF treatment.

It is considered that TLR2-MyD88-NF κ B played an essential role in promoting inflammation. Several studies showed that PM2.5 could work as pathogen-associated molecular patterns (PAMP) combining with pattern recognition receptors (PRR) like TLR2 on the surface of the airway epithelial cells and alveolar macrophages. TLR2 delivered the activated signals to downstream MyD88 and then induced the phosphorylation of NF κ B, which caused inflammatory cytokines synthesis. This signal transduction was generally recognized as an important mechanism of PM2.5-triggered inflammatory injury[26]. However, in the current study, the levels of TLR2, MyD88, and NF κ B phosphorylation of each group are consistent with no significance. The reason for this is because, except for NF κ B related pathway, there are numerous approaches promoting inflammation, such as MAPKs, Smads, and Nrf2 signalling pathways[30–32].

MUC5AC, the primary component of airway mucus, is synthesized and released by goblet cells in the epithelium. We detected by AB-PAS staining that exposure to PM2.5 could significantly exacerbate the goblet cell hyperplasia and MUC5AC release in the airway. The results of MUC5AC protein and mRNA expression in lung tissues came out the same trend. After treatment with MGMD and YFPF, we found that the therapy could reduce the goblet cell proliferation and inhibit MUC5AC expression. Moreover, the effects of YFPF was more significant than that of MGMD. In terms of the mechanism, it has been considered that EGFR-PI3K-AKT signalling pathway is tightly associated with regulating MUC5AC mRNA expression. PM2.5 and PM2.5-induced inflammatory mediators cause excess MUC5AC synthesis via the cascade of EGFR-PI3K-AKT signalling pathway[18]. Western Blot bands manifested remarkable increase in the ratio of phosphorylation of PI3K and AKT. However, phosphorylation of EGFR showed no difference in each group, indicating that EGFR is not the main signal receptor that causes the cascade of PI3K-AKT

in this study. It has been investigated that receptor tyrosine kinase (RTK), G protein coupled receptor (GPCR) and T cell receptor can also bind to extracellular stimulation signals and activate the PI3K-AKT signalling pathway[33]. According to the results of Western Blot, YFPF had the effect of simultaneously inhibiting the phosphorylation of PI3K and AKT, whereas MGMD only showed a significant difference in AKT. After synthesized, MUC5AC protein was packed tightly in granule in the cytoplasm of goblet cell and remained stable until activated by stimuli like NE and MCP-1. MGMD and YFPF could downregulate the expression of NE and MCP-1, but IHC images showed that YFPF had more inhibitory effect on NE and thereby had advantage on suppressing MUC5AC release in turn.

According to TCM theory, the pathogenesis of PM2.5-induced respiratory symptom is “wind evil invading the lung”. YFPF, a classic formula, can dispel wind-evil and consolidate superficies. In recent researches, YFPF was discovered to be effective in restricting inflammation, alleviating allergic response, repairing tight junction of epithelia barrier[34–36]. GMD which comes from experience prescription is mainly applied in various allergic diseases. Previous study suggested that the mechanism of GMD was associated with reducing the production of IgE and suppressing inflammatory cell infiltration[20]. In clinical application, GMD with Qianhu and Jiegeng was more potent in treating allergic symptom for the two herbs could recover the lung function of dispersing and descending. Recent researches also explored that Qianhu and Jiegeng could significantly attenuate eosinophilic airway inflammation, downregulate the expression of inflammatory cytokines, and inhibit the activity of Th2 cells[37, 38].

Conclusions

In conclusion, this research indicated that MGMD and YFPF could significantly attenuate PM2.5-induced type1 and type2 inflammation of lung by regulating the inflammatory mediators. Meanwhile, both two therapies could inhibit the airway mucus hypersecretion through PI3K-AKT signalling pathway. The result suggested that YFPF had more advantage in preventing type1 inflammation and mucus hypersecretion, while MGMD was more beneficial in reducing type2 inflammation. However, more underlying mechanism remains to be explored further.

Abbreviations

PM2.5: fine particulate matter; MGMD: Guo-Min Decoction; YFPF: Yu-Ping-Feng Powder (YFPD); TNF- α : tumor necrosis factor; MCP-1: monocyte chemoattractant protein; TLR2: Toll-like receptor 2; MyD88: myeloid differentiation primary response gene 88; NF κ B: nuclear factor kappa B; ICAM-1: intercellular cell adhesion molecule 1; BALF: bronchoalveolar lavage fluid; MUC5AC: mucin5AC; EGFR: epidermal growth factor receptor; PI3K: phosphatidylinositol- 4,5-bisphosphate 3-kinase; AKT: protein kinase B.

Declarations

Authors' contributions

Changle Zhu and Guo Fang have been involved in rat model establishment and treatment. Feng Feng and Changle Zhu performed IHC, ELISA, qPCR and Western blot analysis. Feng Feng and Meng Yufeng have made contribution to acquisition and analysing data. Feng Cuiling have been involved in designing the study and drafting the manuscript. All authors read and gave final approval for the version submitted for publication.

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Acknowledgements

Not applicable.

Competing interests

These authors have no conflict of interest to declare.

Availability of data and materials

The datasets used in this study are available from the corresponding author upon reasonable request.

Consent for publication

The manuscript is approved by all authors for publication.

Ethics approval and consent to participate

The experimental protocol was approved by the Experimental Animal Ethics Committees of Peking University People's Hospital (No. 2017PHC022).

Funding

This research was supported by the National Natural Science Foundation of China (2017, No. 81673921).

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Figures

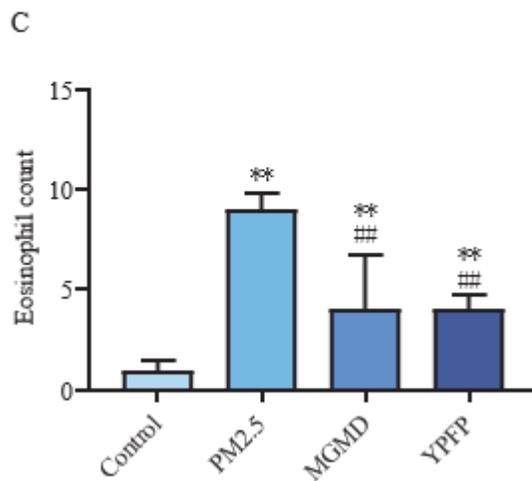
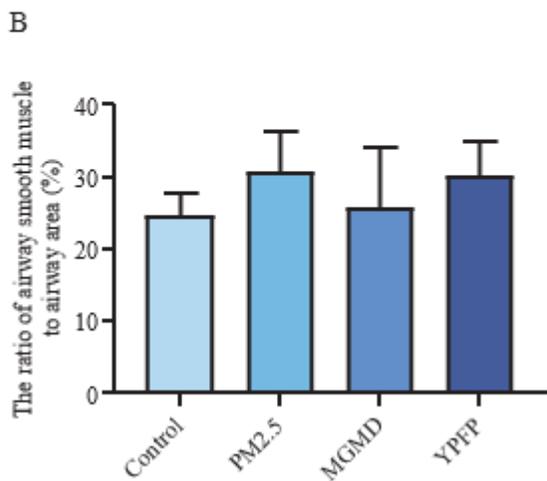
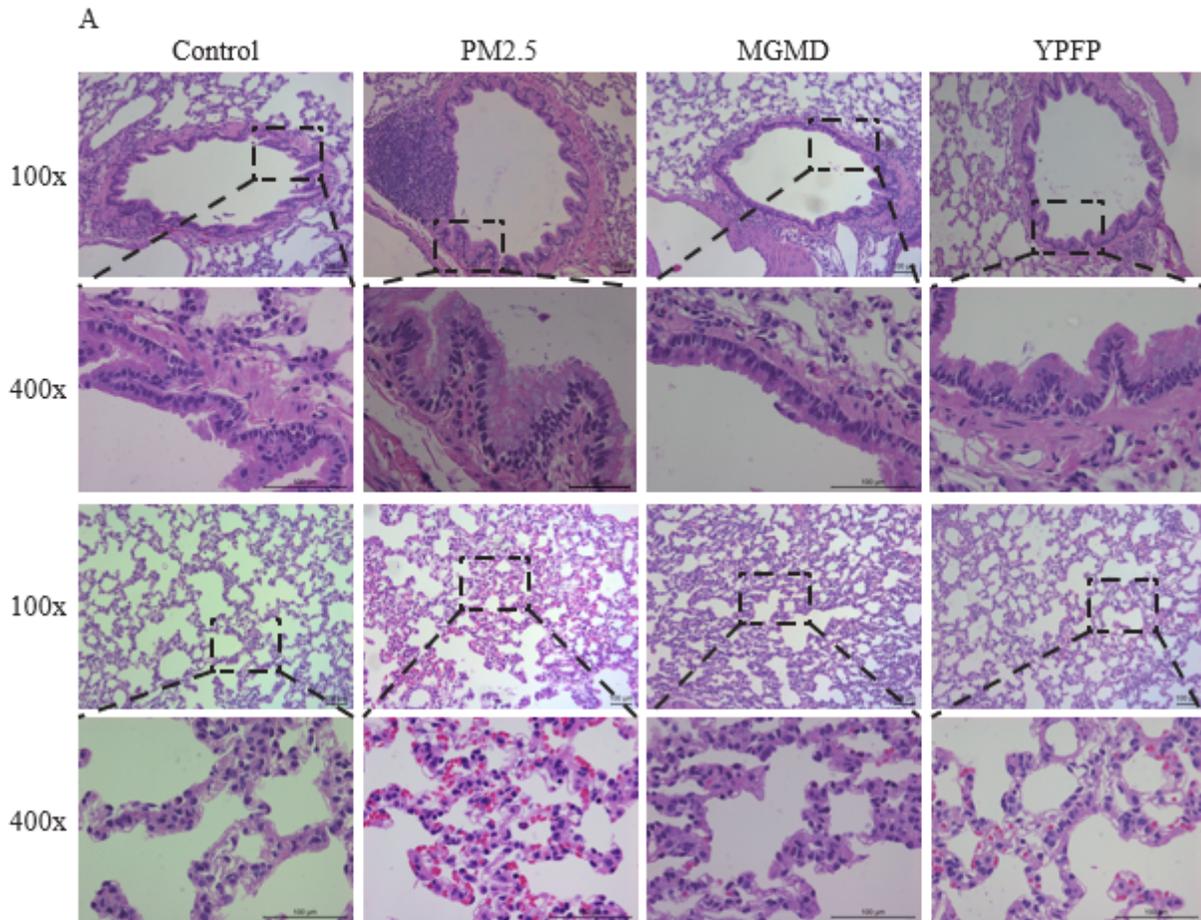


Figure 1

Effects of MGMD and YPFP on the histopathological change, airway smooth muscle and eosinophil of lung in PM2.5 rats. A: Control group displayed normal morphology of airway and alveoli. PM2.5 group showed airway and alveolar structural damage. The lung injury were mitigated in MGMD and YPFP (H&E staining, $\times 100$ and $\times 400$). B: Quantitated the airway smooth muscle by the ratio of smooth muscle area to airway area (n=5). C: The mean count of eosinophils around the airway in MGMD and YPFP groups

showed significantly decrease compared with PM2.5 group (n=5). The value was expressed as mean \pm SD. Applied One-way ANOVA for statistical analysis. **P < 0.01 vs the Control group, ##P < 0.01 vs the PM2.5 group. MGD: modified Guo-min Decoction, YPFP: Yu-ping-feng Powder.

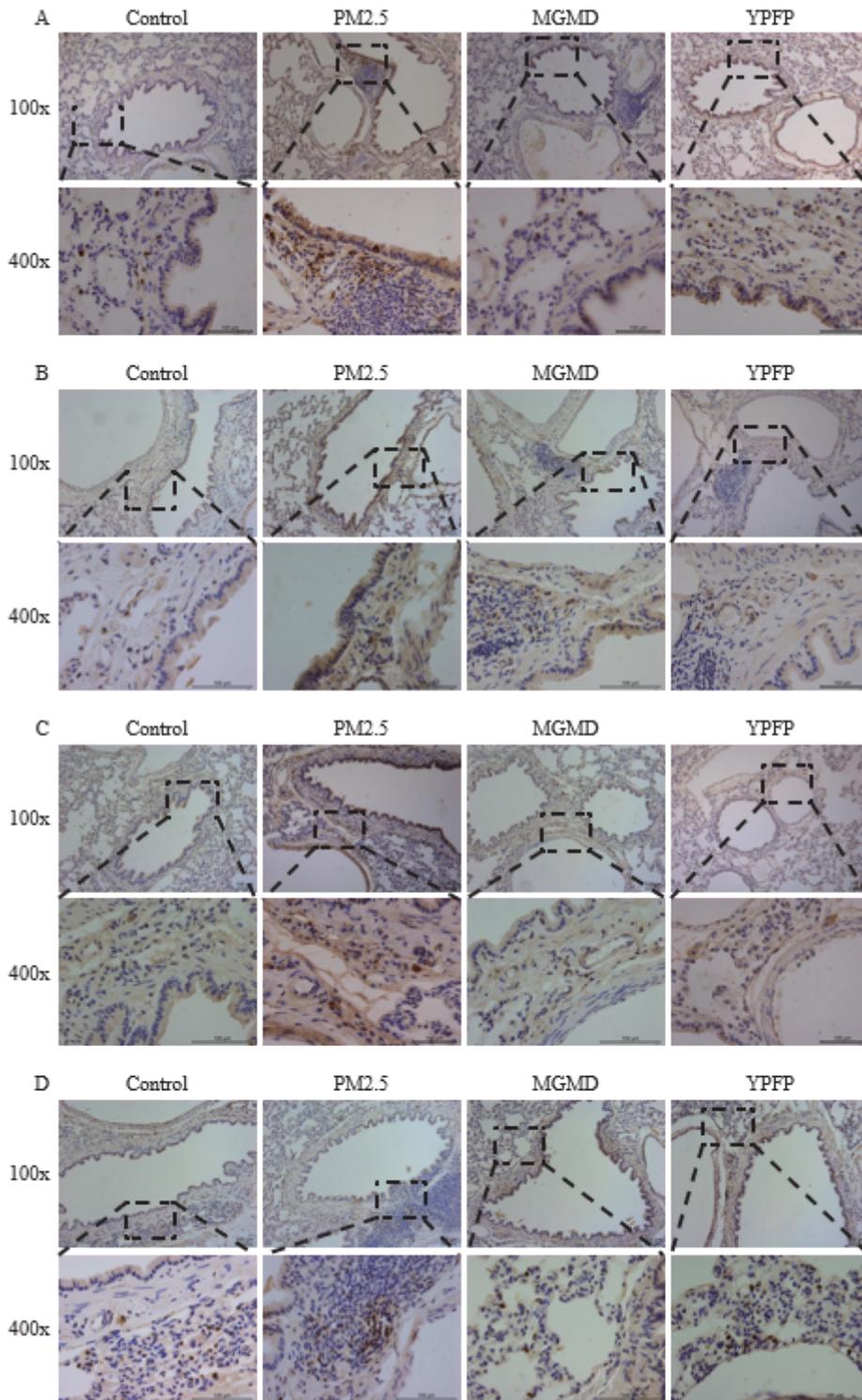


Figure 2

Effects of MGD and YPFP on the inflammatory cells in lung tissues of PM2.5 rats. A: MPO, the marker of activated neutrophils, was stained brown by IHC (magnification $\times 100$ and $\times 400$). B: CD68, the surface

marker of mononuclear macrophage, was stained by IHC staining (magnification $\times 100$ and $\times 400$). C and D: T-lymphocytes which specifically expressed CD4 and CD8 were detected by IHC staining respectively (magnification $\times 100$ and $\times 400$).

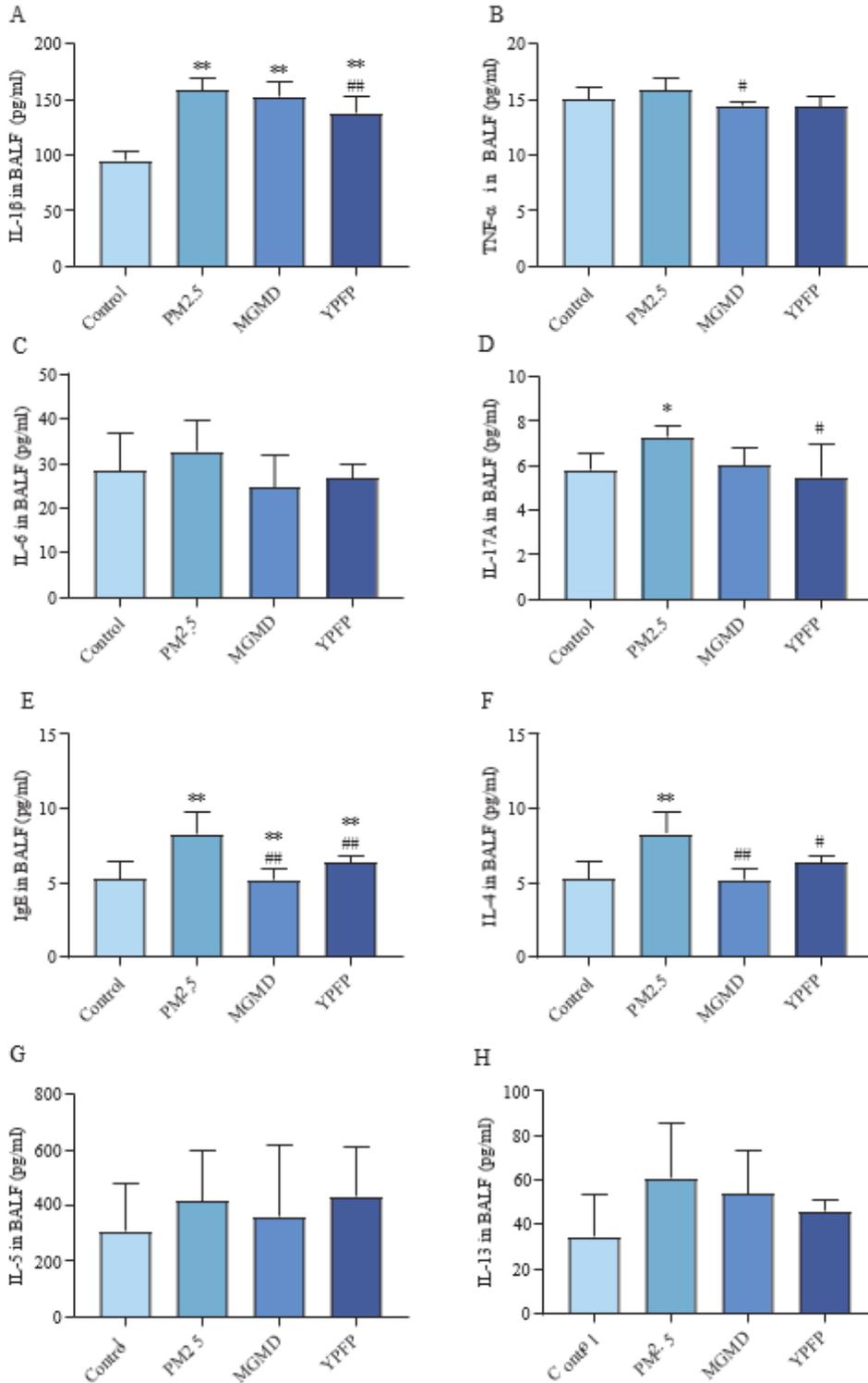


Figure 3

Effects of MGMD and YFPF on the inflammatory cytokines in BALF of PM2.5 rats. A-H: The level of IL-1 β , TNF- α , IL-6, IL-17, IgE, IL-4, IL-5, and IL-13 in BALF of each group. The statistics were shown as mean \pm SD

and analyzed by One-way ANOVA (n=5). *P < 0.05, **P < 0.01 vs the Control group, #P < 0.05, ##P < 0.01 vs the PM2.5 group. MGMD: modified Guo-min Decoction, YPFP: Yu-ping-feng Powder.

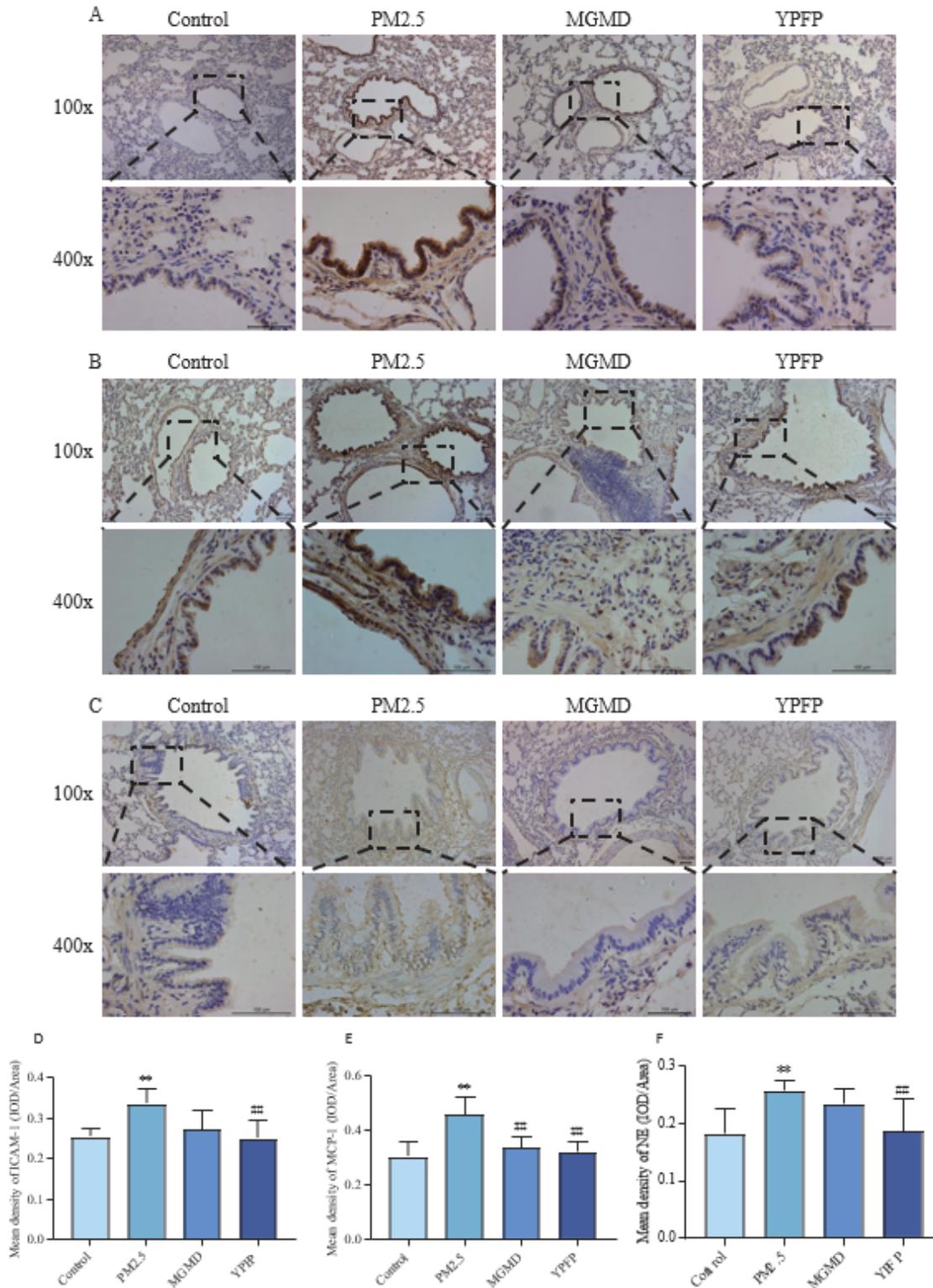


Figure 4

Effect of MGMD and YPFP on the inflammatory mediators in lung tissues of PM2.5 rats evaluated by IHC. A and D: IHC and positive staining quantitation of ICAM-1. B and E: IHC and positive staining quantitation of MCP-1. IHC and positive staining quantitation of NE. The quantitation was estimated by

the proportion of positive IOD to the positive area. The values were presented as mean \pm SD and analyzed by One-way ANOVA (n=5). **P < 0.01 vs the Control group, ##P < 0.01 vs the PM2.5 group. MGMD: modified Guo-min Decoction, YPFP: Yu-ping-feng Powder.

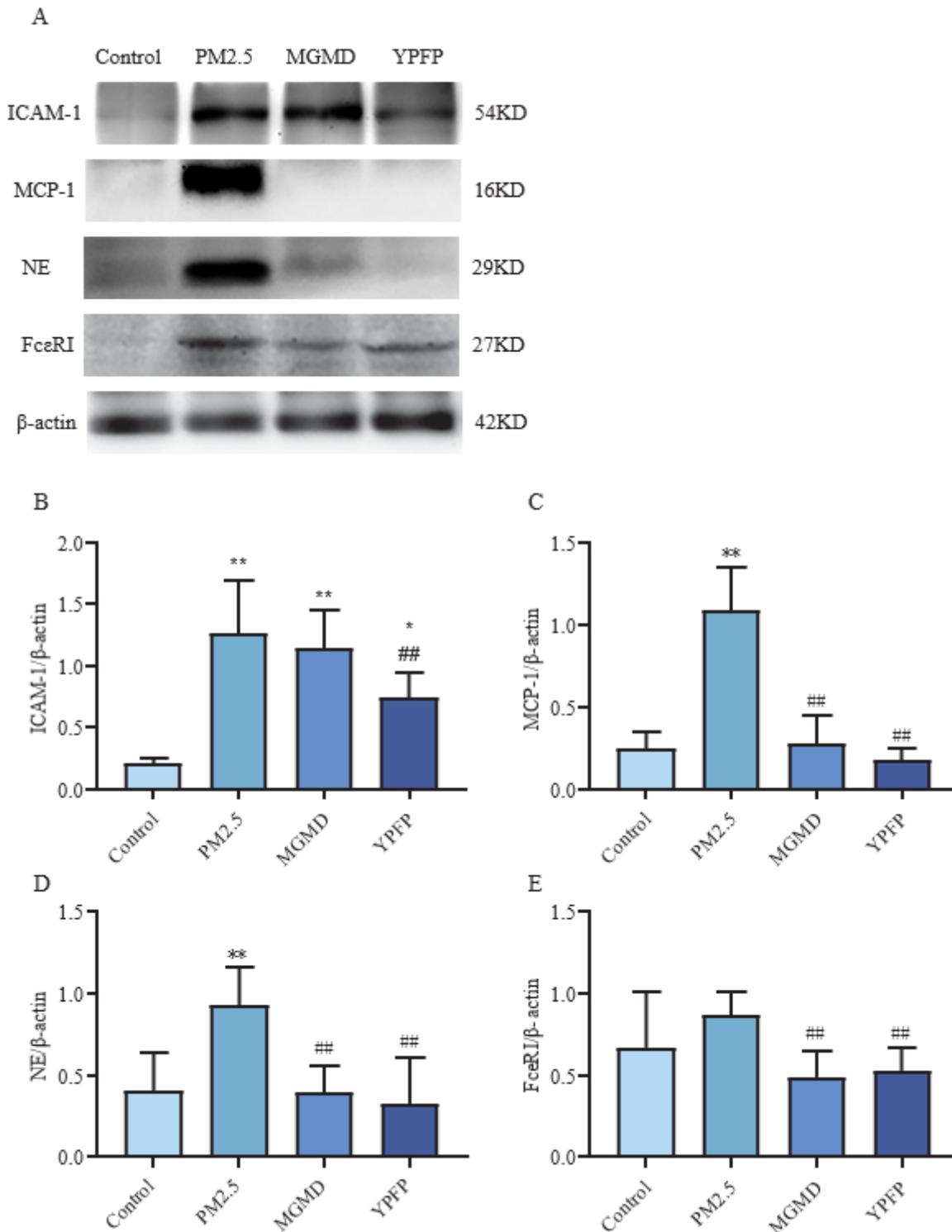


Figure 5

Effect of MGMD and YPFP on the inflammatory mediators in lung tissues of PM2.5 rats evaluated by Western Blot. A: The bands of ICAM-1, MCP-1, NE, FcεRI and β -actin as the internal control. B-E: Estimated

the average optical density of each band. The statistics were expressed as target protein vs β -actin (mean \pm SD, n=5). The statistical significance was analyzed by One-way ANOVA. *P < 0.05, **P < 0.01 vs the Control group, ##P < 0.01 vs the PM2.5 group. MGMD: modified Guo-min Decoction, YPFPP: Yu-ping-feng Powder.

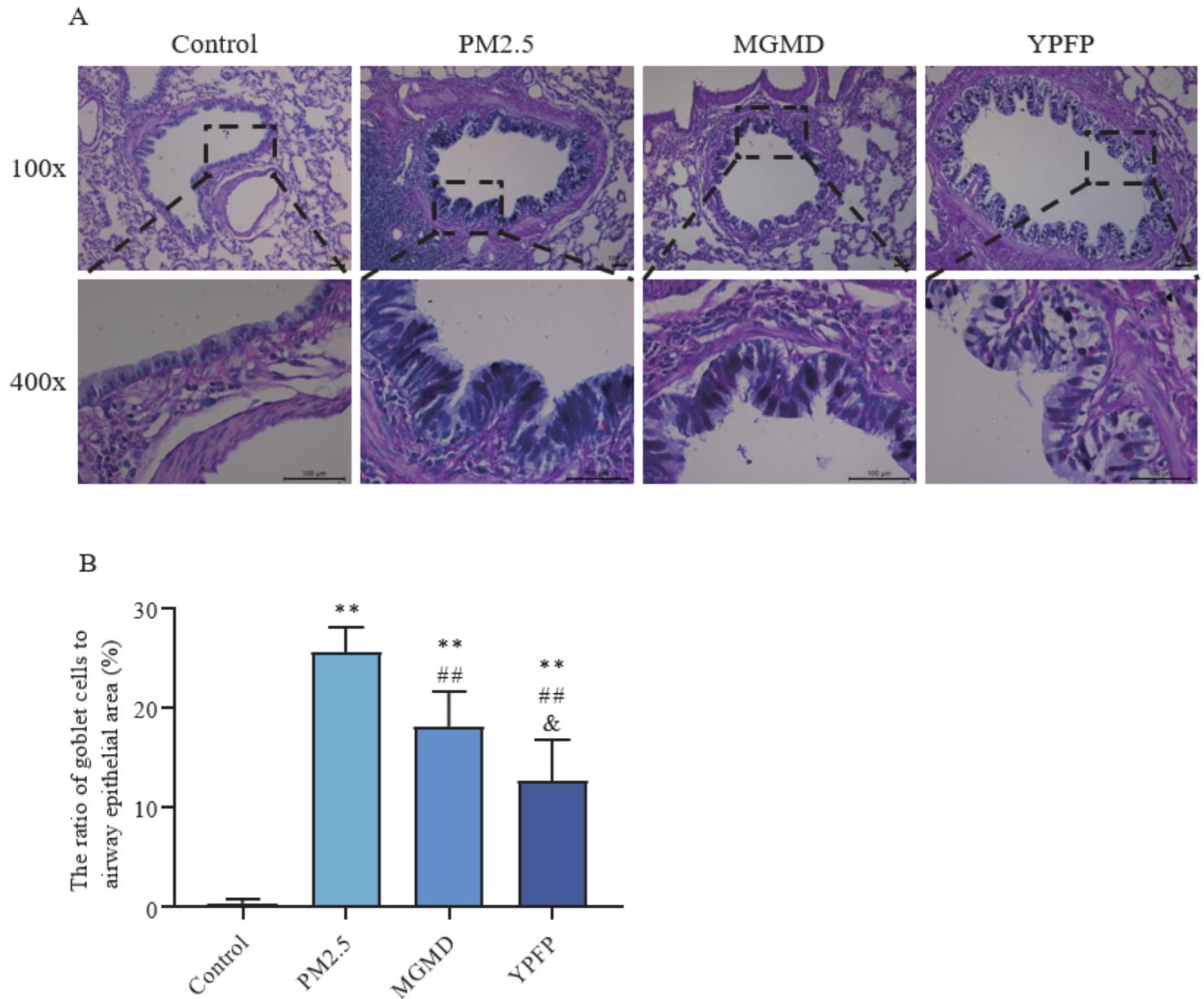


Figure 6

Effects of MGMD and YPFPP on goblet cells in bronchial epithelium of PM2.5 rats. A: The goblet cells was detected by AB/PAS-staining, which was presented deep blue (magnification \times 100 and \times 400). B: Quantitation of goblet cell in the bronchial epithelium was expressed by the ratio of goblet cell area to the airway epithelium area (n=5). The results were analyzed by One-way ANOVA. **P < 0.01 vs the Control group, ##P < 0.01 vs the PM2.5 group, &P < 0.05 vs the MGMD group. MGMD: modified Guo-min Decoction, YPFPP: Yu-ping-feng Powder.

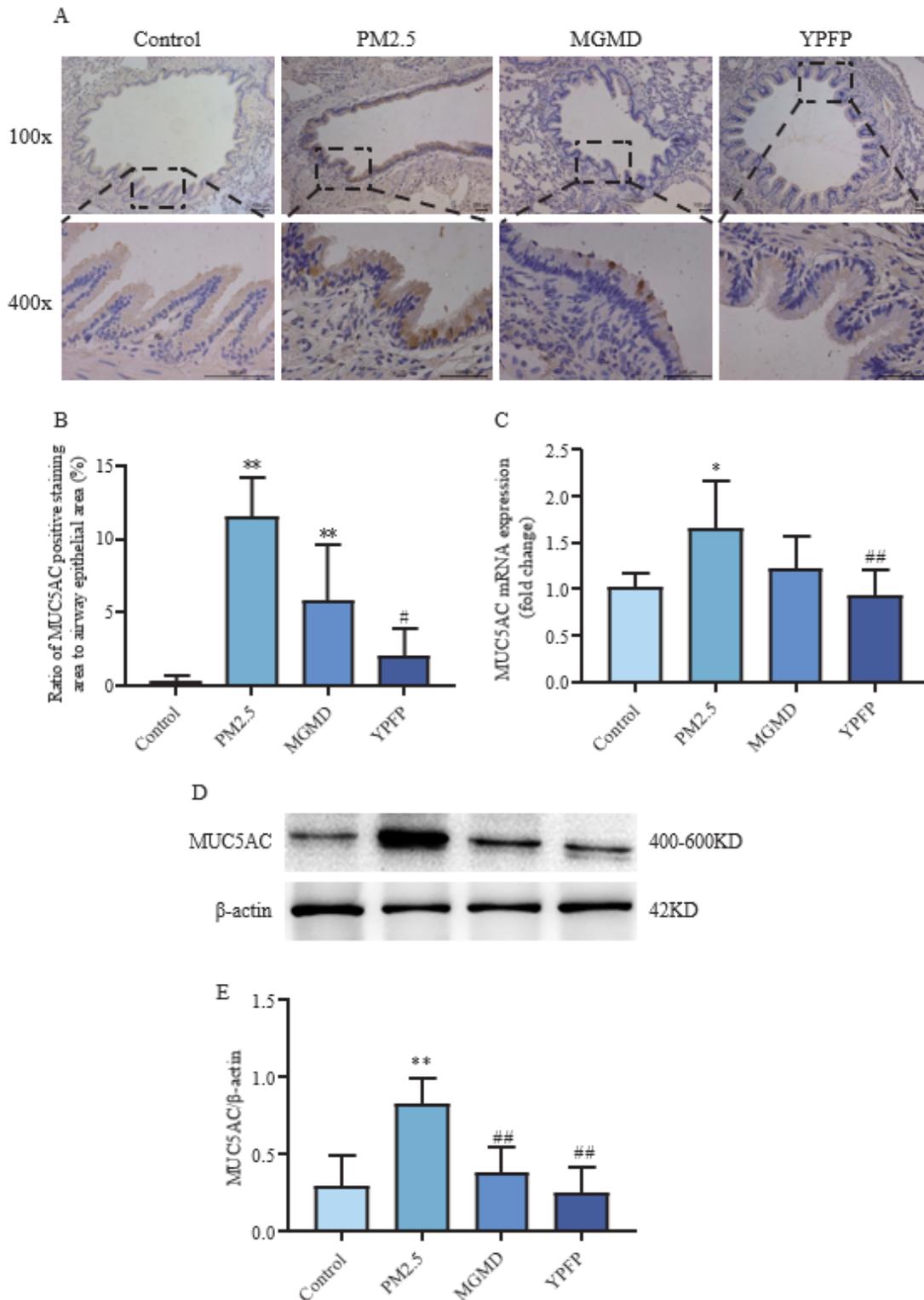


Figure 7

Effects of MGMD and YFPF on the protein and mRNA of MUC5AC in lung tissues of PM2.5 rats. A: The positive IHC staining of MUC5AC in the bronchial epithelium (magnification $\times 100$ and $\times 400$). B: The quantitation of MUC5AC IHC staining was obtained by the ratio of positive area to epithelium area ($n=5$). C: MUC5AC mRNA relative expression of each group. D and E: MUC5AC protein of each group was estimated by Western Blot and normalized to β -actin. The values were shown as mean \pm SD. One-way

ANOVA was used to analyzed statistical significance. *P < 0.05, **P < 0.01 vs the Control group, #P < 0.05, ##P < 0.01 vs the PM2.5 group. MGMD: modified Guo-min Decoction, YPFP: Yu-ping-feng Powder.

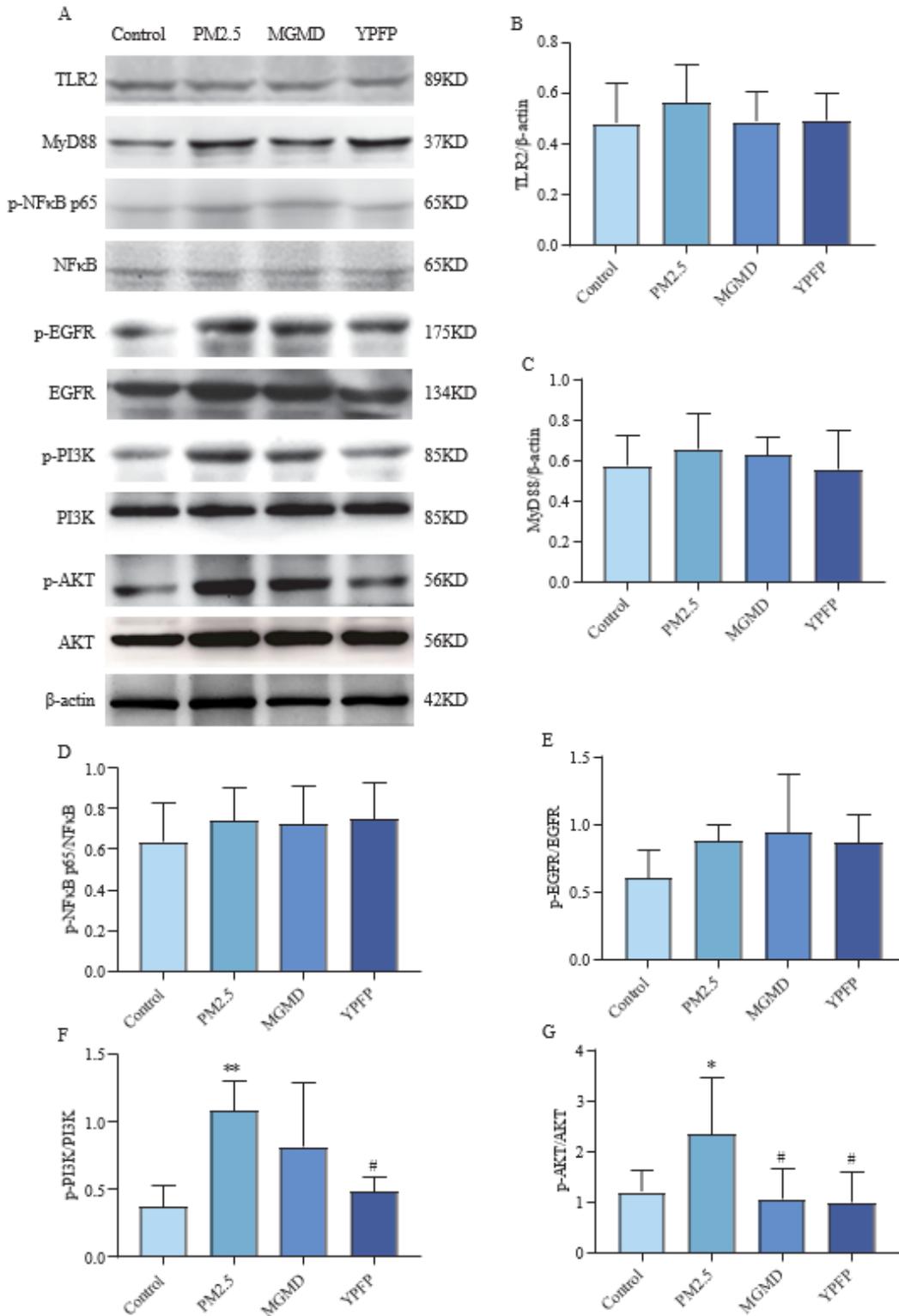


Figure 8

Effect of MGMD and YPFP on the TLR2-MyD88-NFκB and EGFR-PI3K-AKT signalling pathway in lung tissues of PM2.5 rats evaluated by Western Blot. A: The bands of TLR2, MyD88, NFκB, p-NFκB, EGFR, p-EGFR, PI3K, p-PI3K, AKT, p-AKT, and β-actin as the internal control. B-G: Estimated the average optical

density of each band. The statistics were expressed as target protein vs β -actin (mean \pm SD, n=5). Analyzed the statistical significance by One-way ANOVA. *P < 0.05, **P < 0.01 vs the Control group, #P < 0.05 vs the PM2.5 group. MGMD: modified Guo-min Decoction, YFPF: Yu-ping-feng Powder.