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Effects of epigallocatechin-3-gallate on the HMGB1/RAGE pathway in PM_{2.5}-exposed asthmatic rats

YuanZhe Li ^{a,1}, LiXia Chen ^{b,1}, FeiFei Guo ^{b,1}, Yang Cao ^c, Wenjie Hu ^a, Yang Shi ^a, XinChun Lin ^a, Jie Hou ^a, LiPing Li ^a, XianFei Ding ^d, YanJun Guo ^{a,*}

^a Department of Pediatrics, Children's Hospital Affiliated of Zhengzhou University, No.33 Long hu Wai huan East Road, Zhengzhou, 450018, Henan, China

^b Department of Pediatrics, The Third Affiliated Hospital of Zhengzhou University, No.7 Kangfuqianjie Road, Zhengzhou, 450052, Henan, China

^c Department of Pediatrics, Zhengzhou General Hospital, Intersection of Hongda Road and Puqing Road, Jinshui District, Zhengzhou, 450000, Henan, China

^d General ICU, The First Affiliated Hospital of Zhengzhou University, No.1 Jianshe Road, Zhengzhou, 450052, Henan, China

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ABSTRACT

Current studies have shown that long-term exposure to fine particulate matter (PM_{2.5}) can aggravate lung injury in asthmatic children. The HMGB1/RAGE pathway may play an important role, but few studies on the HMGB1/RAGE signaling pathway in PM_{2.5}-induced asthma have been performed. Epigallocatechin-3-gallate (EGCG), which has antioxidant, anti-inflammatory and immunomodulatory effects, has not been examined in studies at home and abroad. In this study, we established an animal model of asthma and observed that the lung tissue was damaged, inflammatory cells infiltrated, bronchial wall thickness (WTt) and bronchial smooth muscle thickness (WTm) increased and the HMGB1 and RAGE mRNA and protein expression increased. The asthmatic rats exposed to PM_{2.5} showed significantly increased lung injury and inflammatory cell infiltration, WTt and WTm further increased, and HMGB1 and RAGE mRNA and protein levels were higher than those in the asthma group. The asthmatic rats exposed to PM_{2.5} were treated with EGCG, which alleviated the lung injury, reduced the number of inflammatory cells, decreased WTt and WTm, and reduced the expression of HMGB1 and RAGE mRNA and protein. The high-dose group showed more significant effects than the other groups. In conclusion, our results suggest that HMGB1 and RAGE are involved in the pathogenesis of asthma. PM_{2.5} exposure significantly aggravated airway inflammation injury in asthmatic rats. EGCG can reduce lung injury and airway remodeling in PM_{2.5}-exposed asthmatic rats and has lung protective effects. The mechanism may be related to regulation of the HMGB1/RAGE signaling pathway. Our results may provide new ideas and methods for the prevention and treatment of PM_{2.5}-induced asthma.

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1. Introduction

Asthma is a heterogeneous disease affected by both heredity and environment [1]. With the acceleration of urbanization and the development of industry in China, environmental pollution is becoming increasingly serious [2]. Fine particulate matter 2.5 (PM_{2.5}) can be suspended in the air for a long time. This material can be accompanied by bacteria, viruses, pollen and other components in the lower respiratory tract and alveoli, causing pulmonary inflammation, which is closely related to the occurrence and

development of asthma [3,4]. High mobility group box 1 (HMGB1) is a DNA binding protein widely distributed in the nuclei of eukaryotic cells. As an inflammatory cytokine, HMGB1 can participate in the occurrence and development of various immune diseases [5].

The receptor for advanced glycation end products (RAGE) is a transmembrane protein of the immunoglobulin superfamily. Studies have shown that HMGB1 can induce respiratory inflammation and oxidative stress injury through the RAGE receptor pathway [6]. Epigallocatechin gallate (EGCG) is the main active and water-soluble component extracted from green tea. It has antioxidant, anti-inflammatory and immunomodulatory effects [7]. EGCG was shown to inhibit the expression of HMGB1 and RAGE [8]. At present, little research has been performed on the HMGB1/RAGE signaling pathway in PM_{2.5}-induced asthma. No reports on the EGCG signaling pathway at home and abroad have been published.

* Corresponding author.

E-mail address: 2289679861@qq.com (Y. Guo).

¹ These authors contributed equally to this work.

In this study, we observed the expression of HMGB1 and RAGE and the protective effect of EGCG on the lungs of a PM_{2.5}-exposed animal model of asthma to provide a theoretical basis for the prevention and treatment of PM_{2.5}-induced asthma in the clinic.

2. Materials and methods

2.1. Animals and reagents

Forty 3-week-old SD female rats (SPF level, Henan Laboratory Animal Center) weighing 52–56 g were used. EGCG (98% purity, Nanjing Biaohe Biotechnology Co., Ltd.), ovalbumin (OVA, Sigma Company, USA), aluminum hydroxide [Al(OH)₃] dry powder (Sigma Company, USA), and PM_{2.5} reagent (Henan CDC) were used. Immunohistochemistry and Western Blot Antibody (Abcam Company, Britain) was obtained. Color reagent, secondary antibodies, tissue protein extraction kit, BCA protein concentration determination kit, chemiluminescence detection kit, TRIzol reagent Ultra-pure RNA Kit, reverse transcription kit and RT-PCR kit were purchased from Beijing Kangwei Reagent Biotechnology Co., Ltd. Primers were from Shanghai Jierui Bioengineering Co., Ltd. IFN- γ and IL-4 (Ray Biotech Company, USA) were obtained. Mage-Pro Plus software (Media Cybernetics, Inc., Maryland, USA) was used to analyze the bronchial wall thickness (WTt) and bronchial smooth muscle thickness (WTm). Animals were fed in the feeding room routinely, freely provided food and drinking water, and fed with standard maintenance feed.

After one week, the animals were used for the experimental study.

2.2. Animal grouping and preparation of animal models

Forty SD rats were randomly divided into five groups with 8 rats in each group: group a (normal control group), group b (asthma group), group c (PM_{2.5} exposure asthma group), group d (EGCG 10 mg/kg intervention group) and group e (EGCG 50 mg/kg intervention group). We prepared rat asthma models by sensitizing and stimulating them with OVA, as described previously by North [9]. In groups b–e, 1 mg of freshly prepared OVA and 2 ml of fluid with Al(OH)₃ (100 mg) were intraperitoneally injected into the rats on the 1st and 8th days. Then, the rats were placed in an airtight container every day and were stimulated with a 1% OVA normal saline solution by a spray nebulizer 30 min each time, from day 15 to day 21. In groups c–e, PM_{2.5} (10 mg/kg) was injected into the trachea from the first atomization to the last, once every other day four times. On this basis, two doses of EGCG were injected separately into the abdominal cavity of each rat in groups d (10 mg/kg) and e (50 mg/kg) 1 h before the first atomization. Rats of group a were treated with saline at the same time. The symptoms of dyspnea, irritability and wheezing in rats showed that the model was successful. All groups of rats were anesthetized by intraperitoneal injection with 10% chloral hydrate (0.3 ml/100 g) 24 h after the last stimulation.

2.3. Histopathology and immunohistochemical staining

The right upper lobes of rats was made into 5- μ m-thick sections. Three paraffin sections were randomly selected for HE staining from each rat. The pathological changes in the lung tissues were observed under high-power microscopy. Airway pathology was evaluated under a microscope. Three paraffin sections were randomly selected for immunohistochemistry from each rat. The positive cells were brown-yellow in the nucleus, membrane or cytoplasm. The distribution of positive cells and the expression of target protein were observed under a microscope.

2.4. Pathological image analysis

Under a light microscope ($\times 400$), 3 complete middle or small bronchi were selected in each rat, and a pathologic image analysis system was applied to collect and measure the bronchial basement membrane perimeter (Pbm), total bronchus area (AO), lumen area (Ai), trachea area in the outer smooth muscle (Amo) and trachea area in the inner smooth muscle (Ami), bronchial wall thickness (WTt), and bronchial smooth muscle thickness (WTm). $WTt = (AO - Ai)/Pbm$; $WTm = (Amo - Ami)/Pbm$.

2.5. Real-time PCR detection

We placed 30 mg of tissue into a mortar, added an appropriate amount of liquid nitrogen to the tissue, ground the tissue into powder quickly, and then extracted the total messenger RNA (mRNA) from lung tissue using TRIzol and an RNA extraction kit. Single-stranded cDNA was synthesized using a reverse transcription kit, and the target gene was amplified using designed primers. HMGB1 upstream primers: 5'-TATCTAAATACGGATTGCTCAGGAA-3', downstream primers: 5'-AGGGACAAACCACAATATAGGAAAA-3', RAGE upstream primers: 5'-TGAAGTACAGCCAATGTCCT-AA-3', downstream primers: 5'-CGAAGCGTGAAGAGACCCGT-3', reference β -actin upstream primers: 5'-C-TGAACCTAAGGCCAACCG-3', downstream primers: 5'-GACCAGAGGCATACAGGGACAA-3'. The PCR system contained the following (25 μ L): 2 $\times$ UltraSYBR Mixture (Low ROX) 12.5 μ L, Forward Primer (10 μ M) 0.5 μ L, Reverse Primer (10 μ M) 0.5 μ L, template DNA 1 μ L, and ddH₂O 10.5 μ L. The PCR conditions were as follows: predenaturation at 95 $^{\circ}$ C for 10 min, denaturation at 95 $^{\circ}$ C for 10 s, annealing at 56 $^{\circ}$ C for 30 s, extension at 72 $^{\circ}$ C for 32 s, and 40 cycles. Melting curve analysis: 95 $^{\circ}$ C for 15 s, 60 $^{\circ}$ C for 1 min, 95 $^{\circ}$ C for 15 s, and 60 $^{\circ}$ C for 15 s. Each sample was repeated three times. Bioer 96 Plus fluorescence quantitative PCR was used to quantify fluorescence. The experimental PCR data were calculated using the $2^{-\Delta\Delta Ct}$ method. The relative expression of the target gene was $2^{-\Delta\Delta Ct}$.

2.6. Western blot

First, 30 mg of right lower lobe lung tissue that was frozen at -80° C was mixed with liquid nitrogen in a mortar, ground into a powder and combined with a mixture of 1 ml tissue lysate and protease inhibitor (99:1). Then, the samples were incubated on ice for 10 min and centrifuged for 10 min, and the supernatant was collected. A BCA protein quantitative analysis kit was used to determine the protein concentration. A 5 $\times$ SDS sample buffer was added to the sample, and the sample size was 25 μ g per pore. A 12% polyacrylamide gel was used for electrophoresis. The voltage for the first 30 min was 80 V, and then, at 1 h, the voltage was changed to 120 V. The total time was 1.5 h. Then, a PVDF membrane was used for protein transfer. The transfer was run at a 220 mA constant current. The transfer time of the target protein and the internal reference GAPDH was 1.2 h. After the transfer was completed, 5% skim milk powder was sealed at room temperature for 1.5 h and then incubated overnight in a refrigerator at 4 $^{\circ}$ C using HMGB1 (1:10000), RAGE (1:1000) and GAPDH (1:1000). The next day, after 1 h of room temperature rewarming, the secondary antibody labeled with horseradish peroxidase was incubated at room temperature for 1.5 h. Exposure was performed after the samples were washed three times with TBST. ImageJ software was used to analyze the gray value of protein bands. The relative expression of the target protein was calculated according to the relative gray value formula (the target protein/gray value of GAPDH).

2.7. Collecting and processing bronchoalveolar lavage fluid (BALF)

Lung tissue was separated, and bronchoalveolar lavage was performed on the left lung three times for each rat. BALF was centrifuged at 4 °C for 10 min at 3000 r/min. The liquid supernatant was collected and stored at –80 °C to test the inflammatory factors. Centrifugally precipitated cells were separated within 2 h and resuspended for smear analysis. The cells were stained with Giemsa staining after air-drying and immediate fixation. The cells were examined and counted under light microscopy. The concentrations of interleukin-4 (IL-4) and interferon- γ (IFN- γ) in BALF were measured by enzyme-linked immunosorbent assays (ELISAs).

2.8. Statistical analysis

The data were calculated and processed by SPSS22.0. Data are shown as the mean $\pm$ standard error ($\pm$) of the mean. Multiple groups were compared with single factor analysis of variance. Single factor analysis of variance was used to compare among groups, and the LSD test was used for comparison between groups. Correlations were analyzed with Pearson's test. The results were considered statistically significant when $P < 0.05$.

3. Results

3.1. Pathological changes

HE staining showed that the bronchial epithelial mucosa of group a was complete and undamaged; there was no obvious inflammatory cell infiltration under the bronchial mucosa and around the lung tissues, and the alveolar wall was smooth. In group b, the epithelial cells of bronchial mucosa were deformed and exfoliated, and the alveolar wall was congested and edema. Inflammatory cell infiltration was observed, and the bronchial mucosa showed hypertrophy and thickening. In group c, inflammatory cells were significantly increased, bronchial epithelial cells showed edema and the alveolar wall showed congestive edema. However, in groups d and e, the alveolar wall edema and inflammatory cell infiltration were reduced, especially in group e. (Fig. 1A). The

analysis of airway morphological parameters under optical microscopy showed that compared with those of the control group, the bronchial wall thickness (WTt) and bronchial smooth muscle thickness (WTm) in the asthma group increased significantly, the thickness of the PM2.5 exposure group increased more significantly, the thickness of the EGCG 10 mg/kg intervention group decreased and that of the EGCG 50 mg/kg intervention group decreased more significantly (Fig. 1B,C).

3.2. Expression of HMGB1 and RAGE

HMGB1 protein can be expressed in the nucleus, cytoplasm and cell membrane of alveolar epithelial cells and bronchial epithelial cells. RAGE is mainly expressed in the cell membrane of alveolar epithelial cells. The expression levels of HMGB1 and RAGE in the lung tissues of group a were weak and were strong in groups b and c; group c had stronger expression than group b. The expression levels of these genes were decreased in groups d and e, especially in group e (Fig. 1D). RT-PCR showed that the expression of HMGB1 and RAGE mRNA in the lung tissues of groups b and c increased, especially in the latter group compared with group a. However, in groups d and e, HMGB1 and RAGE mRNA expression was decreased and was lower in the latter group than the former group. $P < 0.05$ was considered significant (Fig. 1E,F).

3.3. Changes in HMGB1 and RAGE protein expression

Western blot analyses showed that compared with those in group a, the expression levels of HMGB1 and RAGE in the lung tissues of asthmatic rats were higher, and the levels further increased in group b but decreased in groups d and e, with the strongest decreases in group e (Fig. 2A,B,C; $r = 0.847$ $P < 0.01$).

3.4. Total number and classification of inflammatory cells in the BALF

The total number and classification of inflammatory cells in the BALF of groups b and c increased, and the results in the latter group were more obvious. However, in group d, the level was lower than

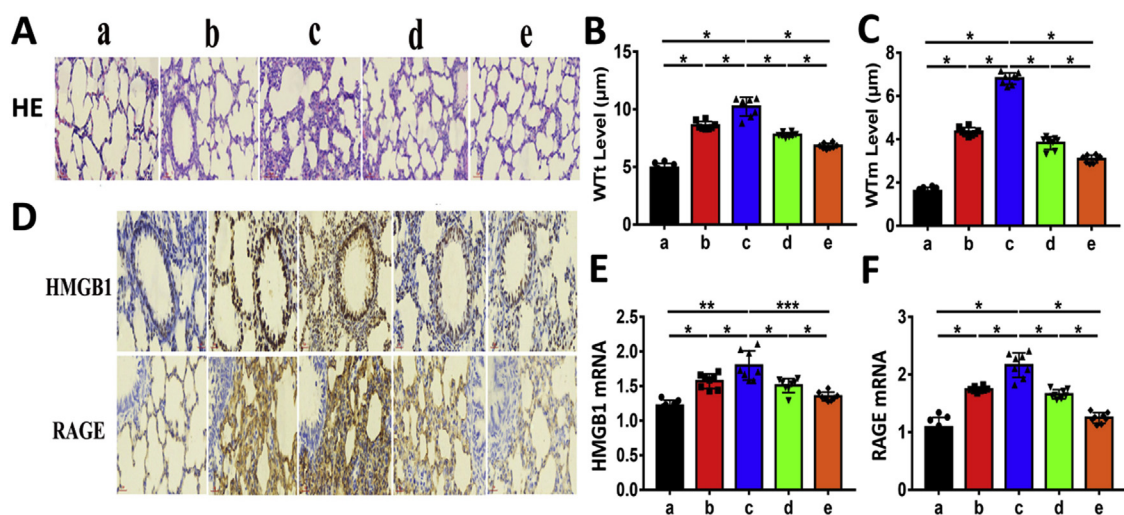


Fig. 1. Pathological (hematoxylin-eosin staining, $\times 400$) and Immunohistochemical (DAB chromogenic, $\times 400$) changes in lung tissue. a: normal control group, b: asthma group, c: PM2.5 exposure asthma group, d: EGCG 10 mg/kg intervention group, e: EGCG 50 mg/kg intervention group. A: Pathological lung tissue varies between groups, after HE staining, cellular morphology was observed. B and C: Analysis of airway morphological parameters between groups under optical microscope, WTt, WTm level; $n = 8$ * $p < 0.05$. D: Immunohistochemical method was used to detect the expression of HMGB1 and RAGE. E and F: RT-PCR analysis for relative expression of HMGB1 and RAGE mRNA in lung tissue. $n = 8$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

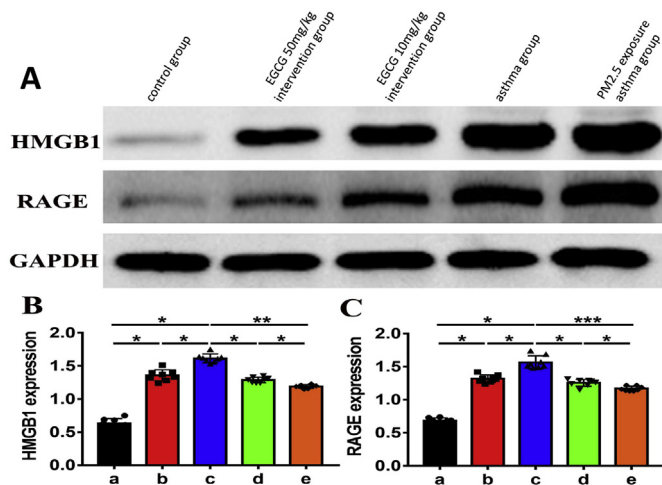


Fig. 2. Western-blot detection of HMGB1 and RAGE protein expression in lung tissue. **A** : HMGB1 and RAGE protein expression changes in lung tissue of rats in each group, $n = 8$. **B** and **C**: a: normal control group, b: EGCG 50 mg/kg intervention group, c: EGCG 10 mg/kg intervention group, d: asthma group, e: PM2.5 exposure asthma group; Western-blot analysis for HMGB1 and RAGE protein expression in each group; $n = 8$. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

3.6. WTt and WTm correlate with HMGB1 and RAGE mRNA and protein expression

According to the Pearson correlation analysis, WTt and WTm were positively correlated with the HMGB1 and RAGE mRNA and protein expression (Fig. 4).

4. Discussion

Airway remodeling is one of the most refractory problems for asthma patients, resulting in irreversible loss of lung function. In recent years, the management model and treatment of asthma have been improved continuously, but the control rate has not been significantly improved. The incidence of asthma has gradually increased in China, which is closely related to environmental pollution [10]. Children are more easily exposed to PM2.5 than adults, and their respiratory and immune systems are immature; thus, the negative effects of PM2.5 on asthmatic children should be closely examined [11]. This study found that HE staining of lung tissue and airway remodeling in group c was more obvious than that in group b. The total number of inflammatory cells, eosinophil ratio and IL-4 concentration in BALF were significantly higher than those in group b, which confirmed that PM2.5-exposed asthmatic rats showed significantly aggravated respiratory inflammation and remodeling.

The HMGB1/RAGE signal has been shown to be involved in the pathogenesis of many chronic airway diseases, especially asthma [12]. In immune responses, extracellular HMGB1 mainly acts as a proinflammatory factor, participates in the process of promoting inflammatory response and oxidative stress, directly participates in the pathogenesis of asthma or acts on dendritic cells, mediates the differentiation of immune cells, and indirectly participates in the pathogenesis of asthma [13]. The release of HMGB1 can also destroy the cytoskeleton, increase the endothelial cell gap, and damage barrier function. Reducing the expression of HMGB1 in the lungs of asthmatic mice can effectively slow the pulmonary inflammatory response [14]. The combination of HMGB1 and RAGE can activate

that in group c and was the lowest in group e (Fig. 3A,B,3C,3D). $P < 0.05$ was considered significant.

3.5. Comparison of IL-4 and IFN- γ in the BALF

The levels of IFN- γ in groups b and c decreased and were more significant in group c than group a. However, IFN- γ increased in groups d and e, more obviously in the latter. The IL-4 levels in groups b and c increased, especially in group c, were lower in group d than in group c and further decreased in group e. (Fig. 3E and F).

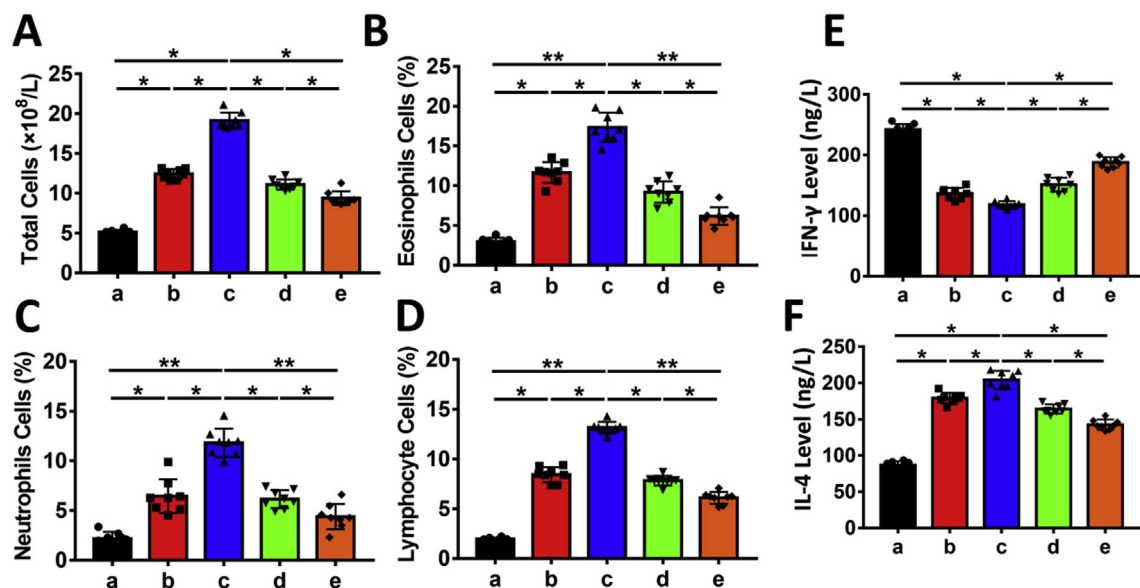


Fig. 3. Total and Classified number of cells in BALF and comparison of IL-4, IFN- γ in BALF of rats in each group. a: normal control group, b: asthma group, c: PM2.5 exposure asthma group, d: EGCG 10 mg/kg intervention group, e: EGCG 50 mg/kg intervention group. **A**: Total cells(%) analysis. **B**: Eosinophils cells(%) analysis. **C**: Neutrophils cells(%) analysis. **D**: Lymphocyte cells(%) analysis. **E**: IFN- γ level analysis(ng/L). **F**: IL-4 level analysis(ng/L) (B). $n = 8$. * $p < 0.05$.

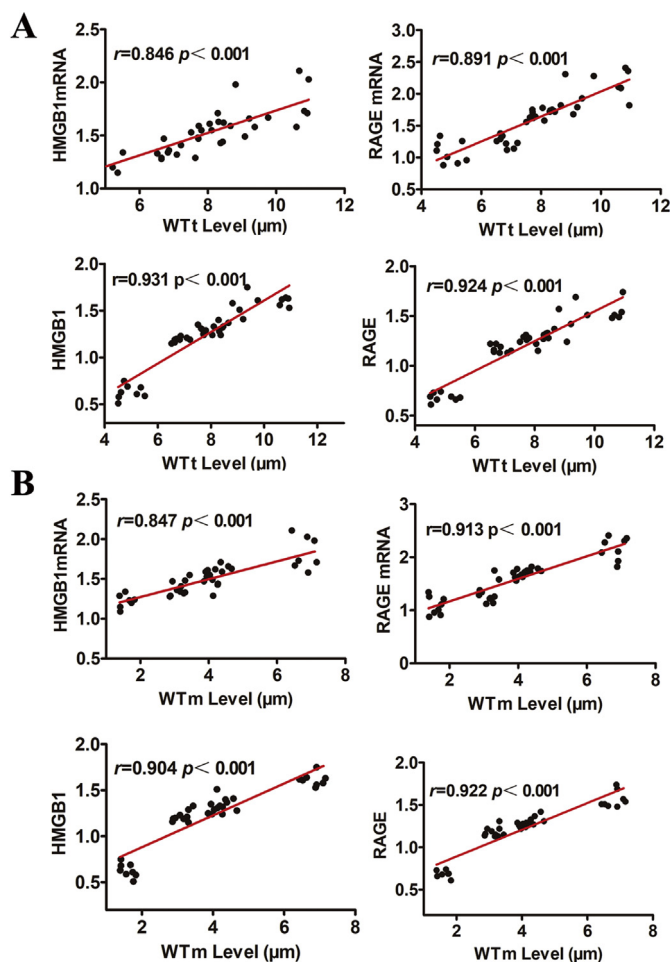


Fig. 4. Analysis of correlation between WTt level and WTm level with HMGB1 and RAGE mRNA and protein expression. WTt was positively correlated with the HMGB1 and RAGE mRNA and protein expression ($r = 0.846$, $r = 0.891$, $r = 0.931$, and $r = 0.924$, $n = 40$, $P < 0.001$) (A). WTm was positively related to the HMGB1 and RAGE mRNA and protein expression ($r = 0.847$, $r = 0.913$, $r = 0.904$, $r = 0.922$, $n = 40$, $P < 0.001$) (B).

NF- κ B, stimulate the production of inflammatory factors such as TNF- α , IL-8, and IL-6, and mobilize chemotactic inflammatory cells to aggravate lung injury [15]. Immunohistochemistry showed that HMGB1 protein could be expressed in alveolar epithelial cells, bronchial epithelial cells, nuclei, cytoplasm and the cell membrane, and RAGE was mainly expressed in the cell membrane of alveolar epithelial cells. The RT-PCR results and Western blot analyses showed that the relative expression of HMGB1 and RAGE mRNA in group c was significantly higher than that in group b and the group a, and pathological sections of the group c showed that lung injury was more serious than that of the other groups. These results confirmed that HMGB1 and RAGE are involved in the pathogenesis of asthma. The rats were continuously exposed to PM_{2.5} for a long time. The expression of HMGB1 and RAGE increased, and the lung injury was severe. The possible mechanism involves HMGB1 activation of the downstream signal transduction pathway through RAGE signaling and promotes the release of cytokines and inflammatory mediators, aggravating lung inflammation and airway remodeling. According to the correlation analysis, WTm and WTt were positively correlated with HMGB1 and RAGE mRNA and protein expression. The HMGB1/RAGE signaling pathway plays an important role in the immune response and airway inflammation. These results support the hypothesis that HMGB1/RAGE signaling

pathways attenuate asthmatic airway inflammation and remodeling. This conclusion is consistent with other findings [16].

Recent studies have shown that immune abnormality is one of the important mechanisms of asthma. The most common immune abnormality in asthma is the proportion and functional imbalance of Th1/Th2 cells; Th2 cells are dominant [17]. This study showed that acute exposure to PM_{2.5} could decrease the level of the Th1-related cytokine IFN- γ and increase the level of the Th2-related cytokine IL-4 in rats. We hypothesized that PM_{2.5} exposure may further promote the immune response of Th2 cells and further aggravate the severity of asthma in sensitized rats. However, whether the HMGB1/RAGE signaling pathway is related to the Th1/Th2 cell ratio requires further study.

EGCG is easily absorbed and has unique biological activities, such as antioxidant, anti-inflammatory and immune regulatory effects [6,18,19]. Recent studies have confirmed that EGCG can induce T cell differentiation. EGCG can play a role in the treatment of asthma [20]. It can inhibit the activity of ZAP70 and then inhibit T lymphocytes, the activities of IL-2 and AP-1 produced by CD3⁺T cells [21], the adhesion and phagocytosis of macrophages by inhibiting the expression of CD11b on the surface of monocytes and macrophages, and the release of many inflammatory factors and neutrophils. In addition, its antioxidant activity is much higher than that of vitamin C and vitamin E [22]. Some scholars have found that EGCG can inhibit the expression of HMGB1 and improve the survival rate of rat septicemia models. EGCG can reduce the aggregation of HMGB1 and its marker IL-6 and interrupt the inflammation mediated by HMGB1 by inhibiting the adhesion of exogenous HMGB1 on the surface of macrophages [7]. In this study, compared with those of group b and group c, the deformity and exfoliation of bronchial epithelial cells and congestion and edema of the alveolar wall in pathological sections of bronchial mucosa were significantly reduced and airway remodeling was alleviated in groups d and e, and these changes were more obvious in the latter group. These results indicate that EGCG, especially high-dose EGCG, could alleviate lung injury caused by PM_{2.5} exposure to asthma. The RT-PCR and Western blot analyses showed that the relative expression of HMGB1 and RAGE mRNA and protein in group d was significantly lower than that in groups b and c, while that in group e was lower than those in the other groups. These results suggest that EGCG can inhibit the expression of HMGB1 and RAGE in alveolar epithelial cells and bronchial epithelial cells. In the BALF of the rats, the total number of cells and the proportion of neutrophils and eosinophils decreased in groups d and e and further decreased in the latter. In addition, the level of IL-4 in group d was lower than that in group c, the level of IFN- γ in group d was higher than that in group c and the level of IL-4/IFN- γ was lower, and the ratio in group e was the lowest. These results suggest that high concentrations of EGCG can significantly inhibit inflammatory cell infiltration and inflammatory factor levels in asthmatic rats, especially PM_{2.5} exposure, and alleviate lung injury.

Conflicts of interest

The authors declare no conflict of interest.

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