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Original Research Article

Effects of Coal Dust Exposure in Eosinophil and Interleukin (IL)-13 on Pulmonary Remodeling in Asthmatic Mice Models

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Abstract

Background: Coal dust is a source of pollution that increases the likelihood of respiratory diseases, including asthma. The combination of asthma and coal dust pollution is associated with inflammation cell activation and pulmonary remodeling. Eosinophils and Interleukin (IL)-13 as inflammatory cells and cytokines also play a role in asthma's pathogenesis and development. This study investigates the impact of coal dust exposure on eosinophil and IL-13 levels in an asthmatic mice-like model on pulmonary remodeling.

Methods: An experiment was conducted on 20-25 g BALB/c mice aged 6 to 12 weeks. The three groups had ten mice each. Groups were sensitized with normal saline, ovalbumin (OVA)-sensitized, and OVA-sensitized + coal dust. The parameters of pulmonary remodeling (the thickness of the epithelium, smooth muscle thickness, the number of goblet cells, and subepithelial fibrosis) and the number of eosinophils were measured with histomorphometry analysis. Total IL-13 concentrations were measured using an IL-13 ELISA kit. The data group of a combination of OVA + coal dust was analyzed using the path analysis method.

Results: From path analysis, it was found that Eosinophils (b=0.006; 95%CI=-2.594 to 2.606; p=0.000) had positive, direct, and statistically significant effects on IL-13. Eosinophils indirectly affected epithelium thickness and subepithelial fibrosis thickness via IL-13. Interleukine-13 had positive, direct, and statistically significant effects on epithelium thickness (b=0.67; 95%CI=-0.129 to 1.471; p=0.010) and subepithelial fibrosis thickness (b=0.682; 95%CI=0.301 to 1.062; p=0.000).

Conclusion: Eosinophils' indirect effect on pulmonary remodeling via IL-13 and IL-13 directly affects airway remodeling, especially epithelium and subepithelial fibrosis components.

Keywords: *Airway remodeling; Asthma, Coal dust; Eosinophil; IL-13*
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INTRODUCTION

Pulmonary remodeling is a cellular and tissue structure transformation caused by the intrusion of foreign substances into the lungs, such as allergens, air pollutants, and others. Asthmatic individuals display airway remodeling. Characterizations of airway remodeling in asthma include epithelial thickness, smooth muscle hypertrophy, goblet cell hyperplasia, and subepithelial fibrosis.[1,2] Changes in pulmonary structure affect mechanical features such as decreased lung compliance, airflow limits, reduced pulmonary function, and increased airway hyper-reactivity compared to persons without health issues.[3]

Pulmonary remodeling in asthma involves many inflammatory mediators. In asthma, eosinophilic and cytokine dominant inflammation from T-helper2/Th2 lymphocytes such as IL-4, IL-5, IL-9, and IL-13.

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Mediators such as histamine, leukotrienes, prostaglandins, and cytokines can cause bronchial spasm, edema, increased mucus secretion, and bronchial smooth muscle contractions in asthma.[3] Eosinophils increase the secretion of proteolytic enzymes, growth factors (transforming growth factor/TGF-β, platelet-derived growth factor/PDGF, fibroblast growth factor/FGF), and oxidative products involved in pulmonary remodeling. Interleukin-13 triggers collagen production and proliferation in fibroblasts and activates TGF-β by upregulating matrix metalloproteinase/MMP.[4] T cells, mast cells, basophils, dendritic cells, and keratinocytes are a few examples of immune and nonimmune cells identified as IL-13 producers. According to some theories, IL-13 acts as an activator, chemotactic, and survival factor for eosinophils. Part of the way that IL-13 encourages eosinophilic inflammation is by increasing the expression of chemokines that attract eosinophils and bind to CCR3. IL-13 promotes leucocytes and resident airway cells to produce CCR4-binding chemokines, which are more prevalent in allergic asthma patients.[5]

Air pollution causes decreased airway function in asthmatic patients.[6] Other studies have reported a relationship between asthmatic exacerbation and air pollution containing PM 2.5, ozone (O3), and nitrate dioxide (NO2).[7] Coal dust is one of the air pollutants containing various organic and inorganic compounds that could trigger inflammation and changes in lung structure, like pulmonary remodeling. Repeated exposure to the airway has been documented to induce harmful respiratory consequences, including asthma.[8] The pathogenesis of the progression of pulmonary remodeling is thought to involve the release of cytokines, chemokines, and growth factors from both inflammatory cells and structural cells due to the induction of exogenous agents. Acute exposure to coal dust induces a pulmonary immune response by increasing the

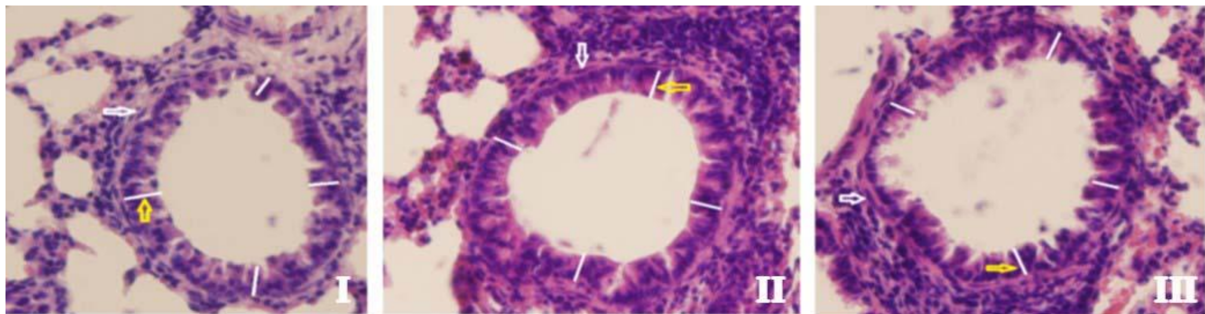

Figure 1. The HE staining of the mouse bronchioles' epithelial histology and smooth muscle (scale 100 µm, 400x magnification); the yellow arrow denotes epithelial thickness. The white arrow indicates smooth muscle. I = Control, II = OVA, and III = OVA + coal dust.

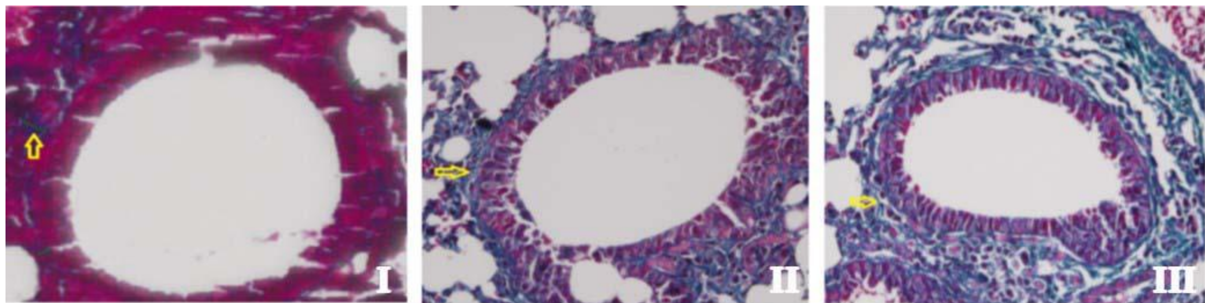

Figure 2. Masson's Trichrome staining of fibrosis subepithelial bronchioles in mice (scale 100 µm, 400x magnification). The yellow arrow indicates the thickness of subepithelial fibrosis. I = Control, II = OVA, and III = OVA + coal dust.

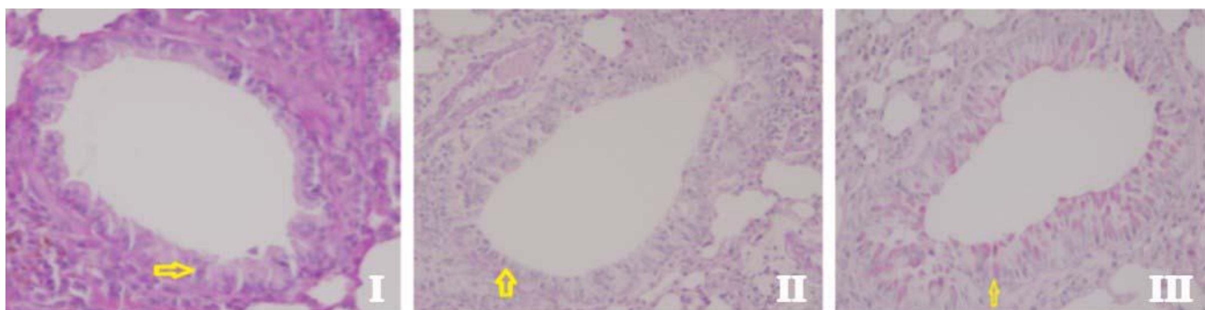

Figure 3. Image of mouse bronchioles goblet cells stained with PAS (scale 100 µm, 400x magnification). The yellow arrow indicates the goblet cell. I=Control; II=OVA; III= OVA + coal dust.

infiltration of leukocytes, neutrophils, and alveolar macrophages.[9,10] In the serum of coal mine workers was found an increase in IL-1β, TNF-α, and IL-6.[11]

Nonetheless, despite the clinical significance of airway remodeling, the mechanism behind its induction to airway epithelial cells, inflammatory cells, and cytokines remains unclear, with only limited studies investigating this topic. An animal model treated with ovalbumin helps research airway remodeling, and subjecting the model to repeated chronic exposure to allergens could lead to chronic inflammation and subsequent airway remodeling. Therefore, this study investigates the pulmonary remodeling process in asthmatic mice exposed to coal dust over a prolonged period. We examined the changes in inflammatory cells such as eosinophil and cytokine (IL-13) in bronchoalveolar lavage and how they impact pulmonary remodeling after exposure to coal dust in animal models of asthma.

MATERIALS AND METHODS

Animal model

Thirty *Mus musculus* (BALB/c), mice from the Farma Veterinary Center in Surabaya, consisting of only females, as they respond better to allergens than males.[12] The inclusion criteria were mice aged 6-12 weeks weighing 20-25 g with a health condition and disease-free (good food consumption, good activity, and no hair loss). All test animals were acclimatized for seven days in the Biochemistry and Biomolecular Laboratory of the Faculty of Medicine, Universitas Lambung Mangkurat. The experimental animals were provided with food and water according to laboratory standards. Experimental animals were divided randomly into three treatment groups, each consisting of 10 mice: (I) negative control; (II) 1% OVA sensitization; (III) OVA 1% sensitization and exposure to coal dust size ≤5µm concentration of 12.5 mg/m^3. Twenty-four hours after the last exposure on day 76, the mice were killed by anesthetized intraperitoneally with ketamine at 150 mg/kg BW and midazolam at 0.5 mg/kg BW. Then, surgery is performed to take bronchoalveolar lavage fluid. An intravenous catheter is inserted through an incision in the trachea, and then the lungs are rinsed with cold saline. The rinse results for eosinophil and IL-13 examination. The lung organ is taken for histomorphometry examination. The ethics committee Faculty of Medicine, Universitas Lambung Mangkurat approved this research with No. 304/KEPK-FK UNLAM/EC/IV/2017).

Ovalbumin (OVA) sensitization

The negative controls received an intraperitoneal (i.p) injection of 1 mg Al(OH)3 in 0.5 ml of normal saline and inhalation of normal saline. The remaining two groups were sensitized and exposed to ovalbumin (OVA) and coal dust. The allergen used was chicken ovalbumin (TCI®). The initial sensitization was performed by administering intraperitoneal injections (i.p) of 10g OVA and 1 mg Al(OH)3 in 0.5 ml normal saline on days 0 and 14. Furthermore, the repeat sensitization involved inhaling OVA 1% using a nebulizer of type NU-017 for 20 minutes, three times per week, for 8 weeks. This process starts from day 21-75.[13]

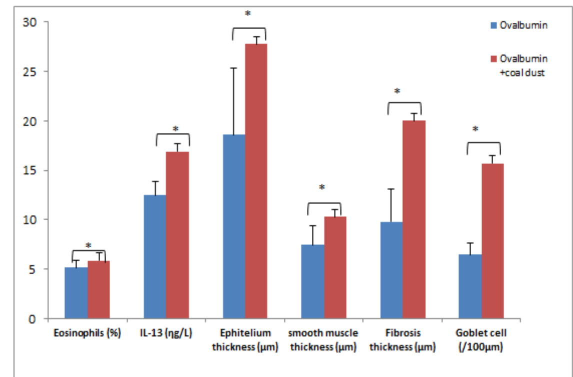

Figure 4. Comparison graph of the number of eosinophils and levels of IL-13 pulmonary remodeling between OVA group with a combination of OVA+coal dust group. Values are presented in mean ± SD. **p* <0.05, statistically t-independent test significant.

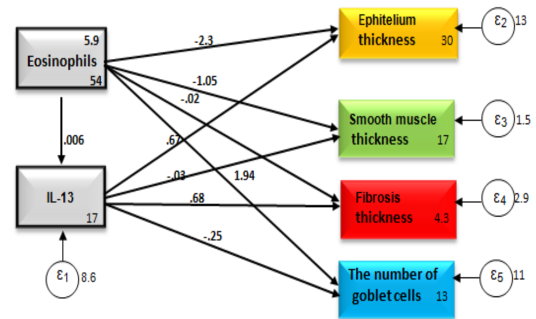

Figure 5: Identification of the path analysis model.

Coal dust creation and exposure

A total of 2 kg of coal were crushed with a pulverizing tool. Making coal dust was conducted in the Carsurin Coal Banjarmasin laboratory. The particles produced were then filtered using a 5 µm PVC chiffon filter to obtain coal dust size ≤ 5 µm.[10]

The exposure to coal dust after four weeks of OVA sensitization. It used a chamber size of 30x30x30 cm^3 with 1.5–2 L/min airflow that resembled the environmental coal mine airflow available at the Biochemistry and Biomolecular Laboratory, Faculty of Medicine, Universitas Lambung Mangkurat. This tool provides an ambient environment containing coal dust exposure to the animal's airway for 1 hour/day for four weeks on days 45-75. An oxygen supply is also provided in the chamber to prevent hypoxia and discomfort. The negative control group was only exposed to room air.[10]

The IL-13 measurement concentrations from lung-homogenate supernatant

Tissues from the left lung were sonicated and mechanically homogenized in a 50 mM Tris-HCl buffered solution at a pH of 7.4. At 40 degrees Celsius, the homogenate was centrifuged at 3200 rpm for two minutes. The lung homogenate supernatant was utilized for the IL-13 determination.[10] Per the manufacturer's instructions, the lung homogenate supernatant was quantified using ELISA kits (Bioassay Technology laboratory/Cat.No. E0019Mo). The Central Biomedical Laboratory, Faculty of Medicine, Universitas Brawijaya, analyzed IL-13. [10]

Table 1. Comparison of the number of eosinophils and levels of IL-13 pulmonary remodeling between the OVA group with a combination of OVA + coal dust group.

Variable (mean ± SD)	OVA	OVA + Coal Dust	*p-value*
Eosinophils (%)	5.167 ± 0.75	5.889 ± 0.782	**0.014**
IL-13 (ηg/L)	12.494 ± 1.382	16.927 ± 3.111	**0.005**
Epithelium thickness (µm)	18.607± 6.727	27.763 ± 4.709	**0.005**
Smooth muscle thickness (µm)	7.444 ± 1.925	10.290 ± 1.549	**0.005**
Fibrosis thickness (µm)	9.79 ± 3,293	20.018 ± 3.843	**0.000**
Goblet cell (/100 µm)	6.444 ± 1.255/10	15,692 ± 2,790/100	**0.000**

Table 2. Results of the analysis of the factor pathways that affect pulmonary remodeling using the eosinophil and IL-13 pulmonary tissue approach

Variable dependent	Variable independent	Coef. Std. Err	95% Conf. Interval		*p*
Direct effects					
Epithelium thickness	←Eosinophils	-2.335	-5.518	0.847	0.150
	←IL-13	0.671	-0.129	1.471	**0.010**
Smooth muscle thickness	←Eosinophils	-1.048	-2.144	0.047	0.061
	←IL-13	-0.032	-0.308	0.243	0.818
Fibrosis thickness	←Eosinophils	-0.017	-1.532	1.498	0.983
	←IL-13	0.682	0.301	1.062	**0.000**
Goblet cells	←Eosinophils	1.938	-0.942	4.818	0.187
	←IL-13	-0.249	-0.973	0.474	0.499
Indirect effects					
Epithelium thickness, Fibrosis thickness	← IL-13 ← Eosinophils	0.006	-2.594	2.606	**0.000**

Log like hood= -112.398; N observation=9; df=19; AIC=262.795; BIC=266.542 (path analysis method STATA MP13)

Eosinophil's lung-homogenate supernatant analysis

The supernatant was obtained from the left lung homogenate for eosinophil examination. One drop of supernatant was placed on the slide, fixed, and stained using Wright-Giemsa stain. One hundred cells were examined on each slide using established criteria to categorize different types of white blood cells. The analysis of eosinophils in the Central Biomedical Laboratory, Faculty of Medicine, Universitas Brawijaya.

Histomorphometry analysis

The histological study was analyzed in the Pathology Anatomy Laboratory, Faculty of Medicine, Universitas Brawijaya. The right lung was preserved with 10% formalin, blocked with paraffin, and microtome to a thickness of 3 mm. Hematoxylin-eosin (HE) staining was utilized to determine the epithelium's and smooth muscle's thickness. The periodic acid Schiff (PAS) technique measured the goblet cell. In contrast, bronchioles subepithelial fibrosis was measured with Massch's Trichome staining collagen deposition by examining blue-stained positive spots chosen beneath the basement membrane. The slides were examined using an Olympus BX51 light microscope at magnifications of 100x and 400x. Three fields of view were observed on each preparation. The photographs were taken with an Olympus DP71 digital camera. Morphometric investigation of structural modifications utilizing Image-Pro Plus 6.1 (Media Cybernetics, Silver Spring, MD).

Statistical analysis

The differentiation between the number of eosinophils and IL-13 level with histomorphometry parameters (epithelial thickness, smooth muscle thickness, subepithelial fibrosis thickness, and bronchial goblet cells) between the two treatment groups (OVA vs OVA + coal dust) was carried out through an independent t-test. STATA/MP 13 (StataCorp LP, College Station, TX, USA) was used to investigate the impact of eosinophil count and IL-13 levels on histomorphometry parameters. The results are presented as the mean ± standard deviation (SD) and β coefficient, with statistical significance at $p<0.05$.

RESULTS

This study revealed that combining OVA and coal dust increased the number of eosinophils, IL-13 levels, epithelial thickness, smooth muscle thickness, subepithelial fibrosis thickness, and bronchial goblet cells compared to OVA alone (Figure 1-3). The epithelial thickness, smooth muscle thickness, subepithelial fibrosis thickness, and bronchial goblet cells numbers of the ovalbumin epithelium + coal dust were significantly higher than the ovalbumin sensitization group with (27.763 ± 4.70 µm vs. 18.607 ± 6.727 µm, p = 0.005), (10.290 ± 1.549 µm vs. 7.444 ± 1.925 µm, p = 0.005), (20.018 ± 3.843 µm vs. 9.79 ± 3.293 µm, p = 0.000) and (15.692 ± 2.790/100 vs. 6.444 ± 1.255/100,p = 0,000), respectively. The t-independent analysis found significant differences (Table 1 and Figure 4).

Eosinophil

Table 1 shows that the number of eosinophils in the ovalbumin + coal dust combination group (5.889 ± 0.782%) was significantly higher than in the ovalbumin sensitization group (5.167 ± 0.75%) (p = 0.014). Table 2 shows that eosinophils do not directly affect pulmonary

remodeling but indirectly via IL-13 on epithelial and subepithelial fibrosis thickness.

IL-13 level

The levels of IL-13 ovalbumin + coal dust group (16.927 ± 3.111 ηg/L) were significantly higher than the ovalbumin group (12.494 ± 1.382 ηg/L) (p = 0.005) (Table 1). Table 2 displays that IL-13 directly influences the thickness of the epithelial and subepithelial fibrosis of the bronchi and bronchioles.

The path coefficient value of each variable shown in Table 1 is more than zero and statistically significant. It shows that the model created in the path analysis in Figure 5 processing with STATA/MP 13 is based on the existing sample data. Therefore, it is unnecessary to re-validate the path analysis model.

DISCUSSION

Effect of eosinophils lung tissue on pulmonary remodeling after exposure to coal dust in an asthma mice model

This study showed that eosinophils do not directly affect lung remodeling. Allergic host reactions involve eosinophils. Degranulation of cationic proteins in cytoplasmic granules is linked to their effector activities. The ability of eosinophils to generate cytokines that mediate a wide range of actions in the local environment has led to the recognition of more diversified functions for eosinophils in multiple tissue areas where they were previously dismissed.[14]

Eosinophils from mice are a source of several cytokines. For instance, mouse eosinophils have mRNA and protein for a ligand that induces proliferation (IL-4, IL-6, IL-10, and TNF-α). Mouse eosinophils produce both type 1 and many chemokines and type 2 cytokines.[15–17] Eosinophils produce several cytokines, such as IL-13, which triggers airway hyperresponsiveness (AHR) and promotes excessive mucus production by stimulating the growth of goblet cells. Mice that did not have eosinophils were shielded from airway hyperresponsiveness (AHR) and excessive mucus production.[18] The Cysteinyl leukotrienes (CysLTs) in asthma significantly exacerbate asthma. In asthmatic patients, there is a substantial association between eosinophil counts and cysteine leukotriene receptor 2 (CyLTR2 M01 V) polymorphism.[19] Goblet cell hyperplasia, collagen deposition, α-smooth muscle actin expression, and profibrotic gene expression are signs of AHR and slow airway remodeling. They can decrease by the leukotriene receptor inhibitor treatment.[20]

Eosinophils secrete several growth factors and fibrogenic mediators to stimulate airway remodeling. Eosinophils are the main source of TGF-β in the bronchial biopsies of asthmatic patients and can stimulate epithelial cells to release various mediators, including TGF-β.[21] TGF- β plays a role in modifying tissues by causing an increase in the number and size of smooth muscle cells, and it controls the activity of fibroblasts that promote fibrosis.[22] Following decreasing eosinophil numbers, TGF-β mRNA expression decreases.[23]

Effect of IL-13 lung tissue on bronchial and bronchiole epithelium thickness after exposure to coal dust in an asthma mice model

The results showed a significant positive effect between IL-13 lung tissue and epithelium thickness. Interleukin 13 increases the bronchial epithelium's and bronchioles' thickness had a log odd 0.67 times (b = 0.67; 95% CI = -0.129 to 1,471; p = 0.010). Each increase in IL-13 lung tissue will increase the thickness of the epithelium by 0.67 times. This study's results are consistent with Everman et al.'s studies that said the direct interaction of IL-13 with epithelial cells increases epithelial cell proliferation in vitro.[24] Other studies mentioned that IL-13Rα2 inhibition would inhibit IL-13 signaling, which causes decreased expression and secretion of growth factors that play a role in repair and are related to airway remodeling.[25]

Effect of IL-13 lung tissue on bronchial and bronchiole smooth muscle thickness after exposure to coal dust in an asthma mice model

The results showed an insignificant influence between IL-13 lung tissue and smooth muscle thickness. Interleukin 13 had a log odd to reduce bronchial smooth muscle thickness and bronchioles -0.032 times (b = -0.032; 95% CI = -0.308 to 0.243; p = 0.818). The findings of this study contrast with those of another study, which stated that the basic fibroblast growth factor (bFGF) is among the factors released by IL-13 in airway smooth muscle cells, promoting smooth muscle cell proliferation.[26] Another review mentioned that IL-13 affects the proliferation of bronchial smooth muscle cells through increased regulation of cysteine leukotriene receptors (cysLT1R) in response to LTD4.[27] Understanding the causes of airway smooth muscle hyperplasia or hypertrophy, regardless of IL-13, can provide insights into how asthma mice model interact with coal dust exposure. Severe pulmonary remodeling due to coal dust exposure in an asthma mice model can trigger smooth muscle cell responses, regardless of IL-13 signals during sub-chronic exposure to coal dust. It may be the involvement of other mediators induced by coal dust, such as IL-8, eotaxin, and MIP-1α reduces the rate of apoptosis of smooth muscle cells in smooth muscle cells, not asthmatics. [28]

Effect of IL-13 lung tissue on bronchial and bronchiole subepithelial fibrosis thickness after exposure to coal dust in an asthma mice model

The results showed a positive and significant effect between IL-13 pulmonary tissue and increased thickness of subepithelial fibrosis after exposure to coal dust in asthma mice models. Interleukin 13 had a log odd to increase thick bronchial and subepithelial bronchial fibrosis 0.68 times (b = 0.682; 95% CI = 0.301 to 1.062; p = 0.000). Inflammation (via eosinophils and B cells) and remodeling (by fibroblasts, airway smooth muscle, dendritic cells, and epithelial cells) are stimulated by IL-13.[29] These results are consistent with previous studies that IL-13 plays a role in the pathogenesis of pulmonary inflammation and alveolar remodeling after exposure to coal dust and provide evidence that polarization of Th2 cells involving IL-13 can support the development of pulmonary fibrosis.[30] Blocking the IL-13Rα2 signal

decreases collagen deposition in bleomycin-induced fibrosis.[31] In vitro, IL-13 activates epithelial cells, and then activation of epithelial cells releases growth factors such as TGF-β and enhances the regulation of collagen type I production.[29] The transition metal content within the coal dust probably causes the effects of coal dust in causing airway remodeling by stimulating cytokines production. According to a scientific study, mineral dust can directly produce fibrosis in the airway wall.[32] Minerals like Cd, Cu, and Se are positively related to asthma exacerbations.[33]

Effect of IL-13 lung tissue on bronchial and bronchiole the number of goblet cells after exposure to coal dust in an asthma mice model

Hyperplasia and hypertrophy of goblet airway cells are features of airway remodeling in asthma related to airway protection and allergen removal.[3] The immunological response is correlated with an augmentation in the quantity of goblet cells of Th2 cells, mainly controlled by IL-13 and IL-4 in asthma. This study showed no positive and significant effect between IL-13 lung tissue and increased goblet cells after exposure to coal dust in asthma mice models. The possible reason for the variation in goblet cell count, regardless of IL-13, could be the interaction between the asthma mice model and coal dust. Coal dust causes pathology in the lungs. Severe pulmonary remodeling due to coal dust exposure in an asthma mouse model can trigger goblet cell responses, regardless of IL-13 signals during sub-chronic exposure to coal dust. This study's results align with those of Ge et al., who stated that other cytokines from Th2 may stimulate goblet cell proliferation, such as IL-9 and IL-5. IL-9 and IL-5 directly induce goblet cell hyperplasia without IL-13 dependence on a mouse model-induced chronic allergen exposure.[34] These data demonstrate that airway goblet cell hyperplasia can be independent of IL-13 associated with the OVA + coal dust combination.

This study established the effects of respiratory coal dust particles on lung epithelial and inflammatory cells in mice asthma models, but it had some limitations. We only examined differentiation between two groups, OVA and OVA + Coal dust group, and did not compare with the coal dust group alone. In future studies, it would be interesting to compare the effects of coal dust only on tissue damage and the biomarkers associated with Eosinophil and IL-13. Our study also was based on a mouse model of asthma; in the future, we plan to confirm the molecular mechanism in human pneumoconiosis.

CONCLUSION

Coal dust is a pollutant particle that can potentially increase the severity of airway remodeling in animal models induced by OVA through the indirect effect of eosinophils via IL-13 and the direct effect of IL-13 on the epithelium and subepithelial fibrosis thickness. The results of this study are hoped to serve as a rationalization for coal pneumoconiosis management and prevention, as well as a starting point for future studies.

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