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RESEARCH ARTICLE

Effects of Coal Dust Particulate Matter Exposure on H_2O_2, MDA, IL-13, TGF-β_3 Level and Bronchioles Sub-epithelial Fibrosis in Allergic Asthma Mice Model

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Abstract

Objective: Pulmonary fibrosis in asthma is marked by a rapid decline in lung function. Unfortunately, the environmental factors aggravating this condition are still poorly understood. To date, the pathomechanism of exposure to coal dust affecting the subepithelial fibrosis in asthma remains unclear. Methods: Twenty-fourth female BALB/C mice were divided into four groups. The first group was control, the second was mice exposed to coal dust particulate matter (PM) (days 46-75), the third was OVA sensitized mice (Initial sensitization on day 0 and 14, re-sensitization days 21-75), and the fourth was made of OVA-sensitized mice and exposed coal dust PM. Results: The results showed coal dust PM significantly decreased levels of IL-13 BAL (p-value=0.001) but significantly elevated H_2O_2 BAL levels (p-value=0.001) and increased sub-epithelial bronchial fibrosis thickness (p-value= 0.000). This was based on the scale of fibrosis (0=<5 µm, 1=5-10 µm; 2=11-15 µm; 3=>15 µm; score 0-1=mild; 1-2=moderate; and 3= severe). The combination of ovalbumin sensitization and PM coal dust caused severe fibrosis (score 3).There was no significant association between IL-13, TGF-β_3, H_2O_2, and MDA BAL with sub-epithelial fibrosis thickness. Conclusions: PM exposure to coal dust may increase sub-epithelial fibrosis of the bronchioles by a mechanism independent of inflammation and oxidative stress.

Keyword: *H_2O_2, MDA, IL-13,TGF-β, Sub-epithelial fibrosis.*

Introduction

Pulmonary remodeling is a change in cell and tissue structure triggered by the entry of foreign objects into the lungs such as allergens and air pollutants Airway remodeling is a condition found in people with asthma, mainly characterized by sub epithelial fibrosis [1]. Changes in pulmonary structure have impacts on pulmonary mechanical properties such as reduced lung compliance [2], air flow limitations, decreased lung function and airway hyper-responsiveness compared to healthy people [3,4].

Many studies have conducted asthma, including the experimental model by stimulating ovalbumin (OVA) induction [5,6,7].The induction of OVA leads to T helper 2 (Th2) activation from cytokine activation in form of IL-13 which in turn stimulate the activation of inflammatory cell, the sub epithelial fibrosis [8].

The sensitization of OVA chicken (serva) through intraperitoneal and inhalation of 1% OVA in 8 ml NaCl conducted by Barlianto [9] established an increase in IL-4 receptor expression, inflammatory cell infiltration and eosinophils. Besides, there aws also change of the airway wall structure of OVA-induced mice compared to the control group.Asthma is often aggravated by continous exposure to allergen, with air pollution as an important factor in its exacerbations [10].

Coal dust particulate matter (PM) is one of the pollutants found in the South Kalimantan, resulting from the coal mining process [11]. If exposures are excessive, lung clearance mechanisms are overwhelmed and dust particles accumulate over time. This causes airflow limitation and the development of interstitial lung disease known as coal workers pneumoconiosis (CWP) which forms progressive massive fibrosis (PMF) [12].

The coal dust PM in the air resulting from coal mining process inhaled cause respiratory tract hypersensitivity, inflammation, and oxidative stress[11]. Coal dust PM entering the respiratory tract induce the alveolar macrophages and release the inflammatory mediator.

This process also produces reactive oxygen species (ROS) which may result to oxidative damage [13]. Reactive oxygen species are produced in response to injuries leading to pulmonary fibrosis. These oxidants activate several genes related to cell growth and death, and fibroblast proliferation [14]. The administration of H_2O_2 in alveolar epithelial and bronchioles epithelial cells may stimulate fibrosis [15].

Oxidants regulate the epithelial-mesenchymal transition (EMT) through activation and expression of Transforming growth factor (TGF) ß1 [16]. Bleomycin-induced pulmonary fibrosis in animal models is associated with increased ROS, protein oxidation, DNA, and lipids [17]. Brass et al suggest that exposure to innate immune stimuli, such as LPS in the environment may exacerbate stable pulmonary fibrosis via independent mechanisms of inflammation and oxidative stress[18].

Sub-epithelial fibrosis begins with epithelial injury followed by the secretion of the profibrotic factors from epithelial cells, fibroblasts and inflammatory cells. Profibrotic factors include TGF-ß and IL-13.

These molecules act as fibroblast and myofibroblasts function regulators, controlling the production of some extracellular matrices such as collagen, proteoglycans, and tenascin [19, 20]. The role of TGF-ß isoform in increasing fibrosis is still controversial because it has an anti-fibrotic properties and anti/profibrotic properties depending on its level. Another study proves airway remodeling involves TGF-ß1 and 2, but not TGF-ß3 in epithelial cell culture[21]. Progressive pulmonary fibrosis is mediated by TGF- isoform 1 but not TGF-ß3[22].

The pathogenesis of pulmonary fibrosis is thought to involve a number of processes leading to alveolar environmental changes and abnormal repair eventually causing fibrosis [23].In this case, the normal and appropriate extracellular matrix, rich in elastin, was replaced with an abnormal matrix rich in fibrillar collagen. These changes impacted pulmonary mechanical properties such as a decreased lung ability to expand (lung compliance) [24].

The novelty of this study was in the fact that the subjects were first made asthmatic and proved the relationship of inflammatory mediators, especially TGF-ß3 and oxidative stress to sub epithelial fibrosis. Therefore, it was important for this study to analyze whether coal dust PM influence an inflammatory response, oxidative stress and pre-existing sub-epithelial fibrosis on allergic asthma mice model.

Material and Methods

Animals Models

Twenty-four *Mus musculus* mice (Balb/c) from Veterinaria Farma experimental animal cages center at Ahmad Yani street Surabaya. The sex of mice selected was female because it has a better response to allergens than male mice[25]. Mice inclusion criteria were: aged 6 to 12 weeks, weight 20 to 25 g in a healthy condition (good appetite and activity, fur did not fall out).

All animals are treated according to the previous procedure [9], and have been approved by the ethics committee of the Faculty of Medicine, University of Lambung Mangkurat (No.304/KEPK-FK_ UNLAM/ EC/ IV/2017).

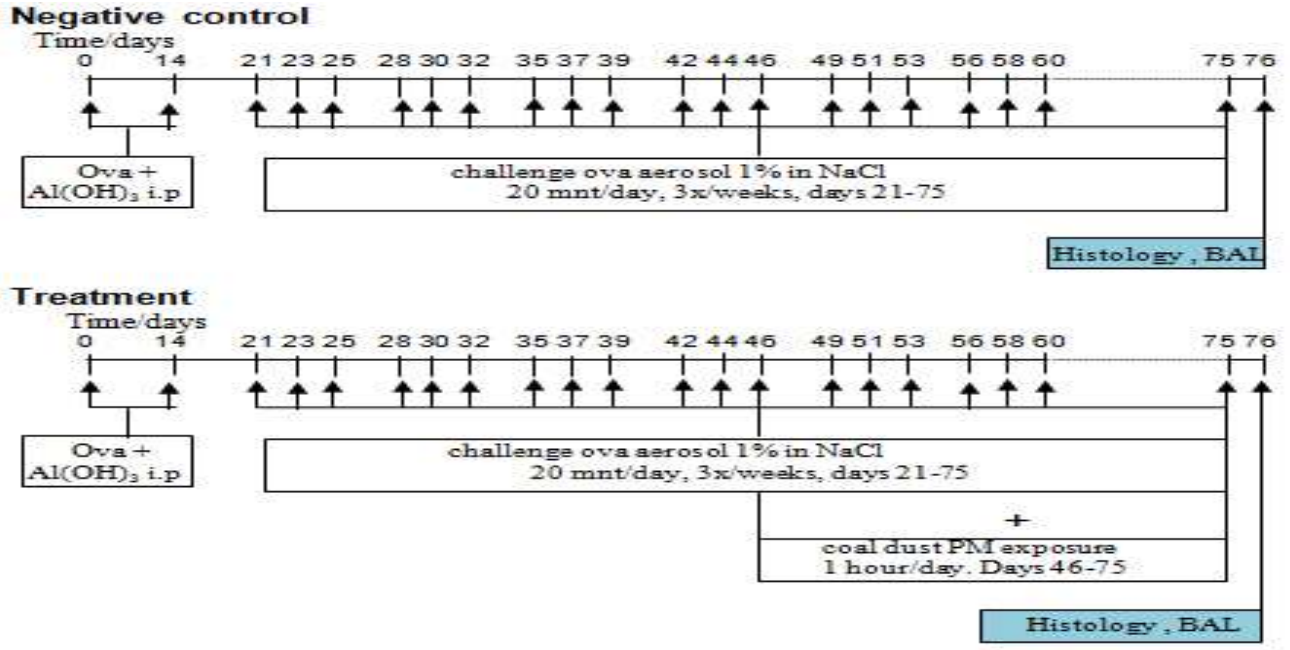

Figure 1: Scheme of the experiment

Animal Models for Ovalbumin Sensitization and Intervention

The test animals were randomly divided into 4 groups (p=6): i), consisting of a negative control group (K); ii) exposed to coal dust PM5 at a concentration of 12.5 mg/m^3 every day for 1 hour/day during 4 weeks (days 46-75); iii) OVA-sensitized 20mnt/day, 3x/week in 8 weeks (day 21-75), iv) and OVA-sensitized (days 21-75) and coal dust PM5 exposure at the same concentration (days 46-75).

The allergen used was ovalbumin chicken (TCI®). Initial sensitization was performed by administration of 10 µg ovalbumin (OVA) and 1 mg Al (OH) $_3$ in 0.5 ml normal saline intraperitoneally on days 0 and 14. Further re-sensitization was done by applying 1% ovalbumin inhalation in 8 ml normal saline with using Omron type nebulizer NU-017 for 20 minutes periodically according to a weekly schedule of 3 times in 8 weeks (day 21-75)(Figure 1). Negative controls were given (i.p) 1 mg Al (OH) $_3$ in 0.5 ml normal saline and normal saline inhalation [14].

Coal dust PM Preparation and Intervention

Coal dust PM was made by Ball Mill, Ring Mill and Raymond Mill in Carsurin Laboratories of Banjarmasin. This coal dust PM was then filtered using Zefon PVC filter membranes (FPVC547-USA) to produce a PM ≤ 5 µm. 12,5 mg/M3 PM5 Coal dust exposure was prescribed based on the previous study[11].

PM exposure was carried out using a 30x30x30 cm^3 coal dust exposure tool designed by and available at Laboratorium of Pharmacology, Faculty of Medicine, and University Brawijaya. This equipment provides an ambient environment that contains coal dust for inhalation by the animal. The airstream of the apparatus was set at 1.5-2 l/min which mimics an environmental air stream.Which provided an ambient environment containing coal dust which will enter the respiratory tract of animals every day for 1 hour during 4 weeks (Figure 1). To prevent hypoxia and discomfort, at chamber oxygen was supplied. Negative controls are exposed only to air filtered in the laboratory.

Measurements of IL-13 and TGF-β$_3$ Concentrations from bronchoalveolar Lavage (BAL)

Twenty-four hours after the last exposure, the mice were anesthetized with ketamine at 150 mg/kgBW and midazolam at 0.5 mg/kg BW intraperitoneally[14]. The left lung was then rinsed three times with 0.9% normal saline using a treeway device. BAL liquids are collected and stored before being processed. The total concentrations of IL-13 and TGF-ß$_3$ were determined using mouse IL-13 ELISA kit (Cat.NoE0019Mo-bioassay Technology Lab) and TGF-ß3 (Cat. No E0854Mo-bioassay Technology Lab).

Measurement of H2O2 and MDA Concentrations from Bronchoalveolar Lavage (BAL)

The concentrations of H_2O_2 and MDA BAL were measured using spectrophotometer (SPECTRO star Nano), using a Hydrogen Peroxide Assay kit (Bio Assay Systems) and a TBARS assay kit (Bio Assay Systems), respectively.

Histomorphometry Analysis

The right lung was fixed with 10% formalin and was made into paraffin blocks, and the blocks cut in 3 µm thickness using a microtome for histological analysis. Masson's trichrome staining was then applied to evaluate the deposition of collagen by analyzing areas positive for fibrosis (stained blue) under the basal membrane. The average value of four fibrotic areas for ten bronchioles per slide (2-4 slides/ animal) was counted using Image Pro Plus 6.1 software (Media Cybernetics, Silver Spring, MD).The fibrosis scale (0= <5 µm, 1= 5-10 µm; 2= 11-15 µm; 3= >15 µm, score 0-1= mild; 1-2= moderate; 3= severe).

Statistical Analysis

The results of data analysis for each group are presented in mean (± SD). The Shapiro Wilk and Levene test were employed to test the normality and homogeneity of the data. A parametric one-way ANOVA test followed by post hoc test LSD 5% was used to examine differences between the group of analyses. LISREL multiple correlation analysis was used to examine the association between inflammation, stress oxidative mediator and fibrosis subepithelial thickness. The statistical analyses were conducted using SPSS version 20.0 (SPSS Inc., Chicago,IL,USA), which a significance level of $p<0.05$.

Results

H_2O_2 and MDA BAL Levels on Group Exposed to Coal Dust PM

The ANOVA statistical analysis showed a difference between treatment groups, and when followed by LSD test, significant difference of H_2O_2 BAL was found to be between group control and coal dust, OVA, OVA+coal dust, and amid coal dust and OVA, OVA+coal dust ($p< 0.05$). This evidence suggests PM exposure to coal dust may increase H_2O_2 BAL levels in OVA group ($p<0.05$). The ANOVA test results report ($p>0.05$), indicating the MDA BAL levels was no significant difference in each group (Table 1).

Table 1: Mean levels of H_2O_2, MDA, IL-13, TGF-β3 BAL and sub-epithelial fibrosis thickness

Experimental group	Control	Coal Dust PM	OVA	OVA+coal dust PM
IL-13 (ηg/L)	55,017±4,897	69,069±6,467*	69,607±7,24*	58,901±5,231°±
TGF-ß3 (ρg/L)	144,571±39,355	201,864±15,088*	223,318±21,565*	223,083±25,130*
MDA (µM)	2,458±0,060	2,503±0,043	2,537±0,072	2,549±0,080
H2O2 (µM)	0,157±0,07	0,336±0,126*°	0,523±0,121*	0,763±0,104*°±
Fibrosis thickness (µm)	6,882±0,935	24,143±6,466*°	8,622±1,246	20,911±3,692*°

Value are mean±SEM (n=6)
*p<0,05, significant change with respect to negative control;
°p<0,05, significant change with respect to OVA sensitization;
±p<0, 05, significant change with respect to coal dust using LSD 5% post hoc test

IL-13 dan TGF-β3 BAL Levels on Group Exposed to Coal Dust PM

The average rate of IL-13 BAL exposed to coal dust PM and OVA desensitization was significantly different compared to the negative control ($p<0.05$). Group 3 exposed to OVA+coal dust PM did not differ significantly with group controls. Besides, the levels of IL-13 BAL in group 3 were significantly lower than in groups coal dust PM and OVA as shown in table1. This suggests there was an effect of coal dust PM in the inflammatory response in mice with existing proinflammatory cytokines. The statistical tests using ANOVA followed by LSD showed significant differences in TGF-ß3 levels between control and treatment groups ($p<0,05$). However, there were no significant differences between coal dusts PM, OVA and OVA+ coal dust PM group as shown table 1. This evidence suggests the coal dust PM exposure had no significant effect on TGF-$ß_3$ level changes in allergic asthma model mice. Sub epithelial fibrosis of the OVA+coal dust PM group was significantly higher than the group controls and OVA group, but lower

than the group exposed to coal dust PM ($p < 0.05$). Based on the scale of fibrosis (in which, 0 = <5 µm, 1 = 5-10 µm; 2 = 11-15 µm; 3 => 15 µm, score 0-1 = mild; 1-2 = 3 = weight), OVA+ coal dust PM caused severe fibrosis (score 3).

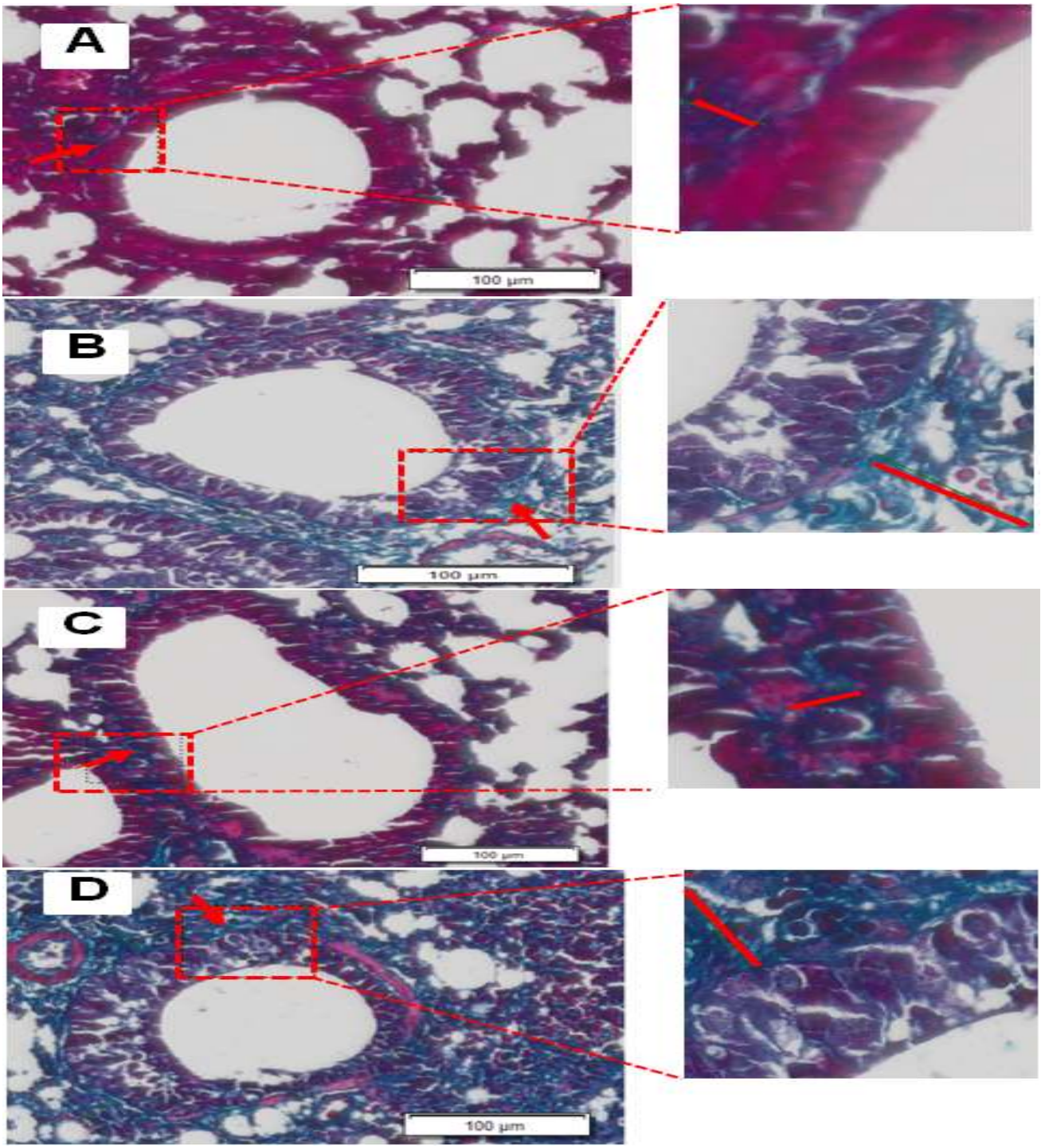

Figure 2. Mice bronchioles subepithelial fibrosis thickness observed using Masson trichome (magnification of 400x). The deposition of collagen by analyzing areas positive for fibrosis (stained blue) under the basal membrane A, control, B, exposure to coal dust PM, C, OVA desentisized, and D, OVA + coal dust PM

Discussion

In the OVA group there was a significant increase in H_2O_2 BAL levels compared to negative controls, indicating it was able to trigger increased production of oxidants in the airways [26]. This is consistent with that found in children where an acute exacerbation of asthma was accompanied by elevated levels of breath H_2O_2. Epidemiological and clinical studies support the association between increased ROS and the pathogenesis of bronchial asthma. The levels of molecules involved in the increase of oxidative stress were found to be higher in the biological samples of asthma patients than in normal controls [27]. Furthermore, patients with severe asthma had higher levels of H_2O_2 in the epithelial lining fluid (ELF) compared to healthy control [28]. Increased levels of H_2O_2 was also found in the group exposed to coal dust PM, proving coal

dust PM could stimulate elevated levels of H_2O_2 BAL. The groups exposed to the OVA +coal dust PM showed significantly increased mean H_2O_2 BAL levels compared to negative control p-value=0.000 ($p<0.05$) and to single exposure group of coal dust or OVA p-value=0.000 ($p<0.05$). Coal dust PM was able to stimulate increased production of H_2O_2 in mice sensitized by ovalbumin in *vitro* studies, showing coal containing pyrite (Fe2S) spontaneously form H_2O_2 and •OH [29]. The formation of •OH compounds in epithelial fluid is suspected due to transition metal content (Cu^{2+}, Fe^{2+}) and quinone in PM [30], with the fenton and haber-weiss reaction converting H_2O_2 to •OH. The high levels of superoxide anion (O_2•$^-$) and SOD activity increased the formation of H_2O_2 [31].

The results of this study showed there was a tendency of elevated MDA BAL levels from all treatments, both exposed to coal dust PM, OVA and OVA+coal dust PM. However, no significant difference in MDA BAL levels in all treatment groups was found. This is in contrast to the results of Bao et al, where ovalbumin sensitization increased oxidative stress and was also characterized by elevated levels of MDA BAL [32].Wistar mice exposed to coal dust PM for 14 and 28 days increased MDA levels of BAL [11]. Increased plasma MDA levels were also found in CWP coal miners compared to healthy controls. Likewise in the Lee et al. study, there was a significant increase in EBC MDA levels in miners with COPD [33]. In this study, exposure to coal dust PM did not significantly influence MDA BAL levels in OVA group, though the H2O2 levels were high in the OVA +coal dust PM group.

There are mechanisms that inhibit peroxidative damage by preventing the decomposition of H_2O_2. The lung has highly specialized and compartmentalized antioxidant defenses for protection against ROS and RNS. These include the following: (1) small-molecular-weight antioxidants (e.g., glutathione), (2) superoxide dismutases (SODs; e.g., mitochondrial manganese SOD [MnSOD], intracellular copper zinc SOD [CuZnSOD], and extracellular SOD [ECSOD]), (3) a group of enzymes which decomposes H2O (numerous glutathione-associated enzymes and catalase), (4) detoxification enzyme systems (e.g., glutathioneS-transferases), and (5) other redox regulatory thiol proteins (e.g., thioredoxin-peroxiredoxin system and glutaredoxins) [34]. GPx catalyzes H_2O_2 and organic peroxide into water or alcohol associated compounds using GSH as a reducing agent [35], limiting the formation of •OH which causes lipid peroxidation. Increased ROS is associated with activation of pro-fibrotic factors, such as TGF-ß, and MMP activation. No significant direct correlation was observed for H_2O_2 BAL with sub-epithelial fibrosis thickness (t count> t table). In this study, it was presumed the effect of H_2O_2 was indirect, as also suggested by Wilgus *et al*, where in the proliferation of fibroblasts was through the activation of TGF-ß1 molecules[36]. Other studies have shown that H_2O_2 interferes with the MMP and TIMP balance, making the formation of ECM more dominant than its decomposition [37].

This was contrary to the research of Reis *et al*., which explains that H_2O_2 decreases inflammation by increasing the apoptosis of eosinophils through caspase activation and cuts remodeling, mucous deposition, cytokine production, and airway hyper-reactivity [38]. The results of this study showed the IL-13 levels in mice exposed to a combination of OVA + coal dust PM were less significant than the controls and considerably lower than OVA or coal dust PM group. The IL-13 BAL levels imply coal dust PM affects the balance of Th1 and Th2 cells after OVA sensitization which was previously dominated by Th2 cells. Cytokines probably released by Th1 cells involved in this phenomenon were IL-2 and IFN-γ.

These proinflammatory cytokines were known to also participate in the development of inflammation. The IL-2 and IFN-γ molecules play a role in tissue injury, and subsequently causes fibrosis [39, 40]. IFN-γ cytokines are known to inhibit the expression of IL-13 *in vivo* and *ex vivo* in immature rats infected with Rhinovirus [41]. A shift in the balance of Th1 and Th2 cells towards Th1 offer protection against asthma and allergies. After exposure to coal dust PM, it is possible that cytokines such as IFN-γ secreted by Th1 cells are involved in the inhibition of IL-13 secretion. IFN-γ is known as the primary effectors of Th1 cells, and plays an important role in Th1 cell differentiation and induces IL-12 production in dendritic cells and macrophages. IFN-γ also provides a direct inhibitory effect on the production of Th2 cell

cytokines, reducing the levels of IL-4 and IL-5. The IFN-γ signal pathway activates T-bet protein, Th1 specific transcription protein, and Th2 suppressant factors [42, 43]. It also suppresses hyper-responsiveness and airway inflammation by induction of Respiratory syncytial virus (RSV), which increases asthmatic exacerbation [44]. However, this study did not find any correlation between IL-13 BAL levels and bronchiolus sub-epithelial fibrosis thickness. This indicates there are other mediators that may influence the progression of sub-epithelial fibrosis such as TGF-ß1 [45]. While this phenomenon is different from other studies, IL-13 was also found to increase in bleomycin-induced fibrosis [46]. The IL-13 is a potential pro-fibrotic factor which suppresses collagen degradation by affecting the synthesis of MMP-1, MMP-2, and MMP-9 which actually reduces the proteolytic degradation of the extracellular matrix, causing an increase in extracellular matrix deposition [47].

It may induce the release of platelet derived growth factor (PDGF) associated with the production of type-I collagen in airway fibroblasts [48]. This molecule triggers the expression of COL1A1 mRNA and the production of collagen protein is increased by 72% compared to controls in dermal fibroblasts. Production of collagen is induced by IL-13 via the Signal Transducer and Activator of Transcription-6 (STAT-6) transcription factor in dermal fibroblasts. The inhibition of STAT-6 results in decreased collagen production. Moreover, no significant difference was found after the administration of IL-13 to the expression of TGF-ß1, whereas even after TGF-ß1 inhibitor administration, the production of collagen induced by IL-13 remained high.

This shows IL-13 triggers the production of collagen not through TGF-ß1 [49].LISREL multiple correlation analysis showed no significant association between TGF-ß3 BAL and sub-epithelial fibrosis. These results prove that TGF-ß3 BAL levels are not involved in the progression of sub epithelial fibrosis. The main sources of TGF-ß proteins include platelets, alveolar macrophages, monocytes, and neutrophils.

This molecule is expressed in injured lung tissue and produced from the inflammatory cells from the lung. Production of TGF-ß increases collagen synthesis. According to Lee *et al*., significantly higher levels of TGF-ß was found in the serum of coal workers with progressed pneumoconiosis compared to CWP without progression[33]. This molecule is still considered important as a growth factor in pulmonary fibrogenesis. The isoforms of TGF-ß include TGF-ß1, TGF-ß2, and TGF-ß3. The TGF-ß1 is heavily involved in fibrosis, though research on the role of TGF-ß2 to fibrosis is lacking. A number of reports indicate TGF-ß2 also promotes fibrosis. In contrast to the two previous isoforms, the role of TGF-ß3 in fibrosis remains unclear [50]. According to Xu *et al*., TGF-ß3 has a protective effect against fibrosis [51].

Khalil *et al* established that TGF-ß3 BAL was involved in pulmonary fibrosis in patients with IPF, though at a much smaller proportion (12%) when compared with TGF-ß1 (69%) and TGF-ß2 (19%) [45]. Several other reports indicate TGF-ß3 is also a fibrogenic, same with the other two TGF-ß [51, 52].To support the latter view, recombinant TGF-ß3 (Avotermin) showed no anti-scarring effects on clinical trials [53]. Studies in mice given HDM showed TGF-ß3 a level is associated with airway remodeling [54]. One of the way of managing the organ injury could be directed toward avoiding the cause(s)[55], herewith the effort to prevent the environmental dust exposure contributing to the pathogenesis of the disease[56].

Conclusion

The exposure to coal dust PM exacerbates ovalbumin-induced bronchiolus sub epithelial fibrosis, though this is not influenced by IL-13 BAL and TGF-ß3 BAL. Exposure of both ovalbumin sensitization and coal dust PM may increase H_2O_2 BAL but not MDA BAL levels. There was no significant association between H_2O_2 BAL levels with sub epithelial bronchiolus fibrosis thickness.

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