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Original Research

Peroxidative index as novel marker of hydrogen peroxide involvement in lipid peroxidation from coal dust exposure

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Lung; Particulatte matter 10

Abstract
This study aimed to elucidate whether inhalation of particulate matter 10 (PM_{10}) coal dust change oxidative stress and peroxidative index in rats. A total of 42 Wistar male rats were randomly divided into seven groups including one control group, three groups exposed to coal dust for 14 days, and three groups exposed to coal dust for 28 days. The three groups at the 14 and 28 days exposures received a dose of coal dust equal to 6.25 mg/m^3, 12.5 mg/m^3, and 25 mg/m^3, respectively. The exposure to coal dust exposure was conducted using equipment that was designed in Pharmacology Laboratory, Medical Faculty, Brawijaya University of Malang, Indonesia. ANOVA test was used to analyze the different level of lipid peroxidation, hydrogen peroxide, and peroxidative index. The number of macrophage was significantly higher in 14 and 28 days coal dust exposure groups compared with control group. The level of lung malondialdehyde, bronchoalveolar lavage malondialdehyde, and peroxidative index were significantly higher in 14 and 28 days coal dust exposure groups compared with control group. The level of bronchoalveolar lavage hydrogen peroxide was not significantly difference in all group. We concluded that inhalation of PM_{10} coal dust increase lung and bronchoalveolar lavage lipid peroxidation, and also peroxidative index of rats.

INTRODUCTION

Inhaled particles to coal dust induce an acute and chronic response that involves a variety of cell types, including alveolar epithelial cells, interstitial fibroblasts, resident and recruited macrophages, and other cells of the immune system. The soluble factor is a mediator of the direct and indirect effects of coal dust, which are involved in the development of acute and long-term lung injury [1]. Mechanisms explaining the fate of particulate matter in lungs are based on intrinsic factors (chemical and morphological) or properties of the dust, as well as host factors, including lung volume, breathing, and depth of respiration [2]. In addition, antioxidant defenses also take part in the process.

The alveolar macrophage is one major cell type in the lung that is constantly exposed to ambient pollutants. Upon contact with environmental particulate pollutants, alveolar macrophages become activated and produce a large quantity of reactive oxygen species (ROS) [3]. Hydrogen peroxide is a major mediator of oxidative stress. Large amounts of hydrogen peroxide can be generated by activated inflammatory cells through the oxidative burst mechanism. Although hydrogen

peroxide is a weak oxidant, it can be converted to highly reactive hydroxyl radicals in the presence of reduced transition metals, such as ferrous (Fe^{2+}) and cuprous (Cu^{1+}) ions [4]. Non-hydroxyl radical mechanisms involve reactions between iron and oxygen to form ferryl or perferryl radials [5]. Malondialdehyde (MDA) is a decomposition product of peroxidized polyunsaturated fatty acids and is widely preferred for the detection of ROS [6, 7]. The peroxidative index is the ratio of lipid peroxidation to hydrogen peroxide levels and determines the involvement of hydrogen peroxide as an inducer of lipid peroxidation.

To date, no studies have assesed the involvement of hydrogen peroxide in lipid peroxidation due to coal dust exposure. We hypothesize that hydrogen peroxide not only acts as a signal messenger, but may also be involved in lipid peroxidation. Accordingly, the objective of this study was to investigate the effect of coal dust exposure on lipid peroxidation and the peroxidative index which can serve as a novel marker of the involvement of hydrogen peroxide in lipid peroxidation.

MATERIALS AND METHODS

Experimental design

Adult male Wistar albino rats weighing 170-200 g were used for the study. The animals were housed in a clean wire cage and maintained under standard laboratory conditions (temperature 25 ± 2°C with a dark/light cycle of 12/12 h). They were fed a standard pellet diet and received water *ad libitum*. The animals were acclimatized to laboratory conditions for one week prior to the experiment. All experimental procedures described were reviewed and approved by the Research Ethics Committee, Faculty of Medicine, University of Brawijaya, Malang, Indonesia. A total of 48 Wistar male rats were randomly divided into seven groups, including one control group, three groups exposed to coal dust for 14 days, and three groups exposed to coal dust for 28 days. The three groups at the 14 and 28 days exposures received a dose of coal dust equal to 6.25 mg/m^3, 12.5 mg/m^3, and 25 mg/m^3, respectively. The exposure to coal dust was conducted using equipment that was designed by and available from Pharmacology Laboratory, Medical Faculty, Brawijaya University of Malang. 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 airstream.

Coal dust preparation and characterization

Coal dust was made from gross coal by pulverizing using a Ball Mill, Ring Mill and Raymond Mill in Carsurin Coal Laboratories of Banjarmasin. This process result coal dust with a diameter of < 75 μm. This coal dust was then filtrated by Mesh MicroSieve (BioDesign, USA) resulting a coal dust < 10 μm of diameter as a respirable particulate matter 10 (PM_{10}). Morphology and size of coal dust was analyzed by a scanning electron microscope at the Physic and Central Laboratory Faculty of Mathematic and Natural Science State University of Malang. The inorganic components of coal dust were then analyzed by X-ray fluorescence.

Tissue bronchoalveolar lavage fluid sampling

The rats were euthanized by inhalation of ether followed by exsanguination. The lungs were harvested from the rats in each experimental group on days 15 and 29 after coal dust exposure, and latter rinsed with physiological saline. All lung samples were stored at −80°C until analyzed. In addition, the trachea was cannulated with plastic catheter attached to a 5-ml syringe, and bronchoalveolar lavage fluid was collected in total volume of sterile saline across two 5-ml samples with gentle massaging of the lungs. The left bronchus was clampes with forceps and the right bronchus was cannulated. Subsequently, 10 ml of heated (37°C) saline was filled and aspirated to and from lung to recover bronchoalveolar lavage fluid. All bronchoalveolar lavage fluid samples were stored at −80°C until analyzed [8].

Malondialdehyde analysis

In the samples (lung tissue and bronchoalveolar lavage) MDA levels were determined using the method of Draper and Hadley [9], based on the reaction of MDA with thiobarbituric acid (TBA) at 95°C. In the TBA test reaction, MDA and TBA react to form a pink pigment with an absorption maximum at 532 nm. The reaction was performed at pH 2-3 at 95°C for 15 min. The sample was mixed with 2.5 volumes of 10% (w/v) trichloroacetic acid to precipitate the protein. The precipitate was pelleted by centrifugation and an aliquot of supernatant was reacted with 0.67% TBA in a boiling water bath for 15 min. After cooling, the absorbance was read at 532 nm. Arbitrary values obtained were compared with a series of standard solutions (1,1,3,3-tetramethoxypropane). Results were expressed as nanomoles per milliliter.

Hydrogen peroxide and peroxidative index analysis

A commercial hydrogen peroxide detection kit (Oxis Biotech international, Portland, OR, USA) was used to measure hydrogen peroxide in bronchoalveolar lavage fluid sampling. Peroxidative index was analyzed by following equation:

$$\frac{\textbf{Lung MDA + BAL MDA}}{\textbf{Hydrogen peroxide level}}$$

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Statistical analysis

Data are presented as mean ± SD and differences between groups were analyzed via ANOVA test using SPSS 16.0 software; $P < 0.05$ was considered statistically significant.

RESULTS

Particle morphology and characteristics

Figure 1 shows the size and morphology of coal dust particulate matter. Scanning electron microscope showed the highest diameter of particle is less than 10 micrometer that confirmed as coal dust PM_{10}. Also, we found ultra- and nano-particles. The morphology of particles demonstrated small agglomerates of particles linked together and formed larger aggregate particles. Besides, X-ray fluorescence has shown inorganic composition of coal dust as iron (36.9%), silicon (17.9%), molybdenum (15%), aluminum (10%), calcium (8.67%), sulphur (4.7%), titanium (3.65%) and several inorganic minerals less than 1% including potassium (0.96%), manganese (0.53%), ytterbium (0.40%), chromium (0.34%), nickel (0.20%), and vanadium (0.16%) [10].

Macrophage number

Table 1 summarizes the numbers of macrophages in lung of control and experimental groups of rats. The number of macrophages was significantly higher in 14 days coal dust exposure groups at doses 12.5 mg/m^3 and 25 mg/m^3 compared with control group. Also, the number of macrophages was significantly higher in 28 days exposure groups at doses 6.25mg/m^3, 12.5 mg/m^3 and 25 mg/m^3 compared with control group. A positive correlation was recorded between macrophage number and dose of coal dust in 14 days ($r = 0.796$; $P < 0.001$) and 28 days ($r = 0.744$; $P = 0.001$) exposure.

Lung lipid peroxidation

The level of lung MDA as oxidative stress marker in lung parenchym of control and experimental groups of rats are shown in Table 2. The level of lung MDA was significantly higher in 14 days coal dust exposure groups at doses 6.25 mg/m^3, 12.5 mg/m^3 and 25 mg/m^3 compared with control group. In addition, the level of lung MDA was significantly higher in 28 days exposure coal dust exposure groups at doses 6.25 mg/m^3, 12.5 mg/m^3 and 25 mg/m^3 compared with control group. There are positive correlation between lung MDA levels and dose of coal dust in 14 days ($r = 0.985$; $P < 0.001$) and 28 days ($r = 0.975$; $P < 0.001$) exposure.

Bronchoalveolar lavage lipid peroxidation

The level of bronchoalveolar lavage MDA was significantly higher in 14 days coal dust exposure groups at doses 6.25 mg/m^3, 12.5 mg/m^3 and 25 mg/m^3 compared with control group. Also, the level of bronchoalveolar lavage MDA was significantly higher in 28 days exposure coal dust exposure groups at doses 6.25 mg/m^3, 12.5 mg/m^3 and 25 mg/m^3 compared with control group as given in Table 3. There are positive correlation between bronchoalveolar lavage MDA levels and dose of coal dust in 14 days ($r = 0.965$; $P < 0.001$) and 28 days ($r = 0.974$; $P < 0.001$) exposure.

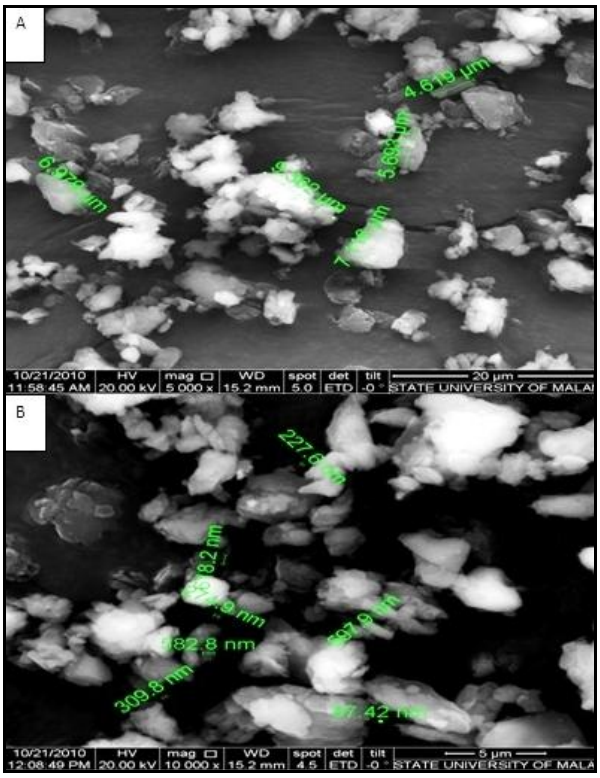

Figure 1. Coal dust particulate matter size and morphology.

Table 1. Macrophage number after 14 days and 28 days of coal dust exposure

Duration	Dose of coal dust			
	0 mg/m^3	6.25 mg/m^3	12.5 mg/m^3	25 mg/m^3
14 days	16.1 ± 5.38	46.18 ± 9.99[a]	58.82 ± 11.46[a]	55.16 ± 3.28[a]
28 days	16.1 ± 5.38	105.38 ± 7.47[a]	91.35 ± 2.75[a]	97.53 ± 1.15[a]

Values are presented as mean ± SD; [a]$P < 0.05$ in comparison with control group.

Table 2. Lung malondialdehyde levels after 14 days and 28 days of coal dust exposure

Duration	Dose of coal dust			
	0 mg/m^3	6.25 mg/m^3	12.5 mg/m^3	25 mg/m^3
14 days	0.0348 ± 0.0079	0.1328 ± 0.0043[a]	0.1749 ± 0.0109[ab]	0.2666 ± 0.0118[abc]
28 days	0.0348 ± 0.0079	0.1716 ± 0.0172[a]	0.2451 ± 0.0085[ab]	0.3048 ± 0.0111[abc]

Values are presented as mean ± SD; P < 0.001 in comparison with [a]control, [b]first dose group, and [c]second dose group.

Table 3. Bronchoalveolar lavage malondialdehyde levels at 14 days and 28 days of coal dust exposure

Duration	Dose of coal dust			
	0 mg/m^3	6.25 mg/m^3	12.5 mg/m^3	25 mg/m^3
14 days	0.0025 ± 0.0004	0.0195 ± 0.0017[a]	0.0368 ± 0.0020[ab]	0.0813 ± 0.0028[abc]
28 days	0.0025 ± 0.0004	0.0703 ± 0.0121[a]	0.1066 ± 0.0097 [ab]	0.1431 ± 0.0091[abc]

Values are presented as mean ± SD; P < 0.001 in comparison with [a]control, [b]first dose group, and [c]second dose group.

Table 4. Hydrogen peroxide levels at 14 days and 28 days of coal dust exposure

Duration	Dose of coal dust			
	0 mg/m^3	6.25 mg/m^3	12.5 mg/m^3	25 mg/m^3
14 days	85.5962 ± 30.6287	116.1214 ± 65.6383	80.7545 ± 42.7774	117.0879 ± 28.8296
28 days	85.5962 ± 30.6287	105.8060 ± 18.8716	160.3301 ± 44.2414	119.0687 ± 51.0478

Values are presented as mean ± SD; no significance among any value (P > 0.05).

Table 5. Peroxidative index in 14 days and 28 days of coal dust exposure

Duration	Dose of coal dust			
	0 mg/m^3	6.25 mg/m^3	12.5 mg/m^3	25 mg/m^3
14 days	0.0004 ± 0.0001	0.0022 ± 0.0022[a]	0.0036 ± 0.0028[a]	0.0031 ± 0.0006[a]
28 days	0.0004 ± 0.0001	0.0024 ± 0.0006[a]	0.0023 ± 0.0007[a]	0.0044 ± 0.0018[a]

Values are presented as mean ± SD; [a]P < 0.05 in comparison with control group.

Bronchoalveolar lavage hydrogen peroxide level

The level of bronchoalveolar lavage hydrogen peroxide was not significantly difference in 14 days and 28 days coal dust exposure groups compared with control group as shown in Table 4. There are no correlation between bronchoalveolar lavage hydrogen peroxide level and dose of coal dust at 14 (r = 0.126; P = 0.641) and 28 days (r = 0.378; P = 0.149) exposure.

Lung peroxidative index

The level of lung peroxidative index was significantly higher in 14 days coal dust exposure groups at doses 6.25 mg/m^3, 12.5 mg/m^3 and 25 mg/m^3 compared with control group. Also, the level of lung peroxidative index was significantly higher in 28 days coal dust exposure groups at doses 6.25 mg/m^3, 12.5 mg/m^3 and 25 mg/m^3 compared with control group as given in Table 5. There are positive correlation between lung peroxidative index level and dose of coal dust at 14 (r = 0.526; P = 0.036) and 28 days (r = 0.805; P < 0.001) exposure.

DISCUSSION

Scanning electron microscope showed clearly the highest diameter of a particle is less than 10 µm that confirmed as coal dust PM_{10}. Also, we found ultra- and nano-particles. The morphology of particles demonstrated small agglomerates of particles linked together and formed larger aggregate particles. The surface area of ultra- and nano-particles was higher compared to µm-particles which determine its reactivity.

Coal dust redox reactivity is determined by its inorganic components and the size of particulate matter. The metal-catalyzed Haber Weiss reaction followed by the Fenton reaction catalyzes the transformation of hydrogen peroxide into hydroxyl radicals [11]. Fenton-type reagents include iron, chromium, nickel, and vanadium. For nickel, the efficiencies are very low due to their relative high oxidation/reduction potential. Other metals, such as chromium, readily react to form hydroxyl radicals. The Haber Weiss-type reaction can involve metals such as iron, chromium and vanadium [12]. Ultrafine particles demonstrate higher redox activity compared to those in larger sizes [13].

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In this study, we found several metals such as iron, chromium, nickel, and vanadium as redox catalyzed reactions. Also, we found ultrafine and nano-particles of size among coal dust particles. The positive correlations between doses of coal dust and lung lipid peroxidation, bronchoalveolar lavage lipid peroxidation, and peroxidative index indicated that active substances in coal dust has redox potency to induce oxidative stress. The higher concentration of active component in coal dust resulted higher oxidative stress.

In the present study, we observed significant increase in macrophage number in the lungs of rats exposed to coal dust. The alveolar macrophage is one of the cell types in the lungs as primary target of coal dust particles [1, 3]. A previous study demonstrated that coal dust increase monocyte chemoattractant protein in lung to differentiate monocytes from circulation into macrophages [14]. In addition, coal dust particles also induce apoptosis of macrophages [15, 16]. The increasing macrophage number in this study is a result due to macrophage differentiation from blood monocytes compared to macrophage apoptosis. The number of macrophages among all doses of coal dust expsoure was not different; this finding indicates that macrophages are in saturated condition with exposure to coal dust particles.

Reactive oxygen species are released during the phagocytosis of inhaled dust particles, and may be generated from particles themselves. The continuous production of ROS may overwhelm antioxidant defenses and may result in oxidative stress leading to cellular damage in the lung [17]. The source of ROS is proposed to derive directly from the coal dust and indirectly from activated inflammatory cells such as alveolar macrophages and polymorphonuclear leucocytes [18]. One of the most frequently used biomarker providing an indication of the overall lipid peroxidation level is MDA [19]. The present study showed elevated lipid peroxidation in lung in agreement with previous works [20, 21]. In addition, the finding of this study demonstrated a significant increase of lipid peroxidation in bronchoalveolar lavage which indicated damage of lipid membrane of epithelial cells and bronchoalveolar stem cells. Bronchoalveolar stem cells localized in the bronchoalveolar duct junction, contain Clara cells and alveolar type II cells. These cells were genuine of lung epithelial stem cells that respond to bronchoalveolar damage [22]. Inorganic components of coal dust such as chromium, nickel, manganese, vanadium have oxidative properties to increase lipid oxidation [23-25]. Iron and titanium were catalyses for hydroxyl radical formation [26]. Also, aluminum is able to react with membrane lipids to promote propagation process of lipid peroxidation [28, 29]. Increased level of lipid peroxidation may cause reduction in the protective response of lung epithelial stem cells to bronchoalveolar damage.

The severity of lipid damage is related to the concentration of the oxidants in the tissue, and hence, to the efficiency of lipid repair mechanisms. The concentration of active metals and inhibitors also determines the severity of lipid damage. Hydrogen peroxide is involved in the oxidative degradation and oxidation of lipid peroxidation [29]. Also, the intracellular accumulation of hydrogen peroxide contributes to oxidant signaling. Much of the damage done by hydrogen peroxide *in vivo* is due to the production of hydroxyl radicals in a series reactions catalyzed by metals. In the present study, we observed no significant increase of hydrogen peroxide in bronchoalveolar lavage compared to control. Broncholalveolar lavage hydrogen peroxide indicates the level of extracellular hydrogen peroxide, which act more as a pro-oxidant than signaling molecule. The main sources of hydrogen peroxide produced in broncholalveolar lavage are xanthine oxidase activity, neutrophils, macrophages and other epithelial cells [30-32]. Degradation of hydrogen peroxide level was conducted by myeloperoxidase, glutathione peroxidase, and catalase [33]. The level of hydrogen peroxide was higher at dose 6.25 mg/m^3 but decrease at dose 12.5 mg/m^3, then increase at dose 25 mg/m^3 of 14 days exposure. The level of hydrogen peroxide was also fluctuative in 28 days exposure. This fluctuative level is a result of the decomposition rate (Fenton reaction, myeloperoxidase, glutathione peroxidase, catalase) and production rate (superoxide dismutase activity, xanthine oxidase activity, neutrophils, macrophages). Iron in coal dust has a dual function as lipid peroxidation initiator and catalyzing of catalase. Manganese catalyses for superoxide dismutase activity.

In the result section, we found that the level of hydrogen peroxide is not significantly different. The level of MDA is result of MDA production and MDA degradation in low pH condition. We use the ratio of MDA:H_2O_2 to know the involvement of hydrogen peroxide bronchoalveolar lavage fluid on series reaction of MDA production and MDA degradation, not acting in oxidant signaling. The level of peroxidative index increased significantly in coal dust exposure groups compared with control group which indicated that the action of hydrogen peroxide as an oxidant is higher in coal dust exposure groups compared with control group. In conclusion, inhalation of PM_{10} of coal dust increases lung and bronchoalveolar lavage lipid peroxidation, and also peroxidative index of rats.

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CONFLICTS OF INTEREST

None declared

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