# Oxydative stress in rats caused by coal dust plus cigarette smoke

## Nia Kania*, Bambang Setiawan**, and H.M.S. Chandra Kusuma***

*Department of Pathology Anatomy, Ulin General Hospital, Medical Faculty, Lambung Mangkurat University, Banjarmasin
**Department of Medical Chemistry, Medical Faculty, Lambung Mangkurat University, Banjarbaru
***Department of Paediatric, Saiful Anwar General Hospital, Medical Faculty, Brawijaya University, Malang

**Correspondence**
dr. Nia Kania, SpPA(K)
Department of Pathology Anatomy, Ulin General Hospital, Medical Faculty, Lambung Mangkurat University
Jl. A Yani Km.2 No.43
Banjarmasin-South Kalimantan
Email: kaniazairin@yahoo.com

## ABSTRACT

Coal dust and cigarette smoke are pollutants found in coal mines that are capable of inducing oxidative stress, the effects of which on blood malondialdehyde (MDA) level and serum superoxide dismutase (SOD) level are still unknown. The purpose of the present study was to evaluate the effect of coal dust and cigarette smoke on levels of MDA and SOD in rats. An experimental study was done on Wistar male rats divided into the following groups: control (C), coal dust exposure (14 days) (CDE), cigarette smoke exposure (14 days) (CSE), coal dust exposure (7 days) followed by cigarette smoke exposure (7 days) (CDE+CSE), cigarette smoke exposure (7 days) followed by coal dust exposure (7 days) (CSE+CDE). All exposures increased MDA levels and decreased SOD activity significantly between groups (p=0.000). All exposure groups had significantly increased blood MDA levels, compared to the control group, although there was no difference between CSE + CDE and CDE + CSE. For SOD levels, all exposure groups had significantly decreased the SOD levels compared to control. But there were no significant differences between CSE vs CDE and CDE + CSE vs CSE + CDE. We conclude that exposure to cigarette smoke significantly increases blood MDA level and decreases serum SOD activity, which was not found in exposure to coal dust. Combined exposures also increase blood MDA level and decrease serum SOD activity significantly.

**Key words:** Coal dust, cigarette smoke, malondialdehyde, superoxide dismutase, rats

## INTRODUCTION

Coal mine dust is a heterogenous and complex mixture of more than 50 organic and inorganic compounds and their oxides. Minerals contained in coal dust include silicates, oxides, carbonates, sulfites, and sulfates. A number of particles are respirable, and therefore increase the risks of disease in coal miners.[1] The diseases among the coal miners do not only comprise pulmonary disorders, such as pneumoconiosis, progressive fibrosis, chronic bronchitis, and accelerated loss of pulmonary function,[2] but also cardiovascular disease

(CVD). An epidemiological study in Appallachian mines found a increased risk of CVD (OR=1.22; 95%CI=1.14-1.30), coronary heart disease (CHD) (OR=1.29; 95%CI=1.19-1.39) and heart attacks (OR=1.19; 95%CI=1.10-1.30).[3]

Oxidative stres is defined as a condition marked by an imbalance between free radicals and antioxidants on the cellular or individual level. Oxidative damage is one of the results of this imbalance, comprising oxidative modification of cellular macromolecules.[4] Several transcription factors that are sensitive to changes in redox status, are activated and coordinate certain biological responses. Low-grade oxidative stress induces transcription of nuclear factor-erythroid related factor 2 (Nrf2), for gene transactivation of the enzymatic antioxidant superoxide dismutase (SOD) as a frontline antioxidant in the defense to oxidative stress.[5,6]

Habitual cigarette smokers are frequently found among coal miners, causing these individuals to be burdened by oxidative stress from coal dust and cigarette smoke. Coal dust particles deposited in the alveolar epithelium are phagocytosed by alveolar macrophages that subsequently increase release of $H_2O_2$ and $\bullet O_2^-$.[7] Cigarette smoke contains free radicals in the tar and gas phases. The tar phase contains $10^{17}$ radicals per gram, which are detectable by electron spin resonance (ESR), are very stable, and persist for hours. The gas phase contains $10^{15}$ radicals per exhalation, consist a shorter half life radicals than the tar phase radicals.[8]

The study by Armutcu et al. demonstrated increased serum malondialdehyde (MDA) levels in rats exposed to coal dust.[9] The study by Junior et al. showed an increase in plasma thiobarbituric acid reactive substance (TBARS) level and a decrease in SOD activity in residents of coal districts and in coal miners, compared to controls.[10] Ulker et al. found increased serum SOD in active coal miners and those on leave, in comparison to controls.[11]

The study by Murarescu et al. evaluated increases in serum MDA levels in rats exposed to cigarette smoke.[12]

On the basis of above mentioned studies, there no studies have been conducted on the combined effect of exposures to coal dust and cigarette smoke. The meeting of two free radical molecules results in termination of their radical status and the formation of neutral compounds. In addition, the reaction velocity of free radicals determines the triggering effect on oxidative damage in biomolecules or reactions with enzymatic antioxidants. This study examined the effect of coal dust and cigarette smoke on levels of MDA and SOD in rats.

## METHODS

### Research design

This was an experimental study, using male Wistar rats weighing 200-250 grams, obtained from the Pharmacology Laboratory, Faculty of Medicine, Brawijaya University, Malang.

### Experimental animals

The experimental animals were assigned to the following groups: control (C); coal dust exposure (14 days) (CDE); cigarette smoke exposure (14 days) (CSE); coal dust exposure (7 days), followed by cigarette smoke exposure (7 days) (CDE + CSE); and cigarette smoke exposure (7 days), continued with cigarette smoke exposure (7 days) (CSE + CDE). There were five rats in each group.[13]

### Preparation of coal dust

Coal dust was prepared by pulverizing gross coal with Ball Mill, Ring Mill and Raymond Mill at Carsurin Coal Laboratories, Banjarmasin. The resulting coal dust was <75 μm in diameter, and subsequently further screened using MicroSieve (BioDesign, USA), producing coal dust of <10 μm diameter as respirable particulate matter. The coal dust

characteristics were analyzed by scanning electron microscopy and X-ray fluorometry at the Physics Laboratory, Malang State University.

**Exposure to coal dust**

Exposure to coal dust was performed by means of a coal dust exposure device of 0.5 m$^3$ capacity, designed and provided by the Pharmacology Laboratory, Faculty of Medicine, Brawijaya University. This device works on the principle of providing an ambient environment containing coal dust as particulate matter, which can be inhaled into the airways of the experimental animals. The air flow through the blower is 1.5-2 liter/minute, corresponding to the air flow in collieries. The dose of coal dust exposure had been determined by preliminary studies to be 12.5 mg/m$^3$ for a high dose.[14]

Cigarette smoke exposure was performed by means of a smoking pump apparatus, designed and provided by the Pharmacology Laboratory, Faculty of Medicine, Brawijaya University. Five rats were placed into the exposure box, then the cigarettes were lighted and the cigarette smoke was introduced into the box, the exposure lasting five minutes.

**Parameter measurements**

Following a 14-day exposure, the rats were anesthesized in plastic jars containing cotton wool drenched in ether. Section was performed on rats with beating hearts by opening the abdomen, cutting the ribs, and opening the thoracic cavity to find the heart. Blood was obtained from the heart to be used for parameter measurements. The parameters measured were blood malondialdehyde (MDA) level and serum superoxide dismutase (SOD) activity, by means of a colorimetric method developed in the Pharmacology Laboratory, Faculty of Medicine, Brawijaya University, Malang.

**Ethics**

This study was approved by Ethics Local Committe in Faculty of Medicine Lambung Mangkurat University, Banjarmasin, South Kalimantan.
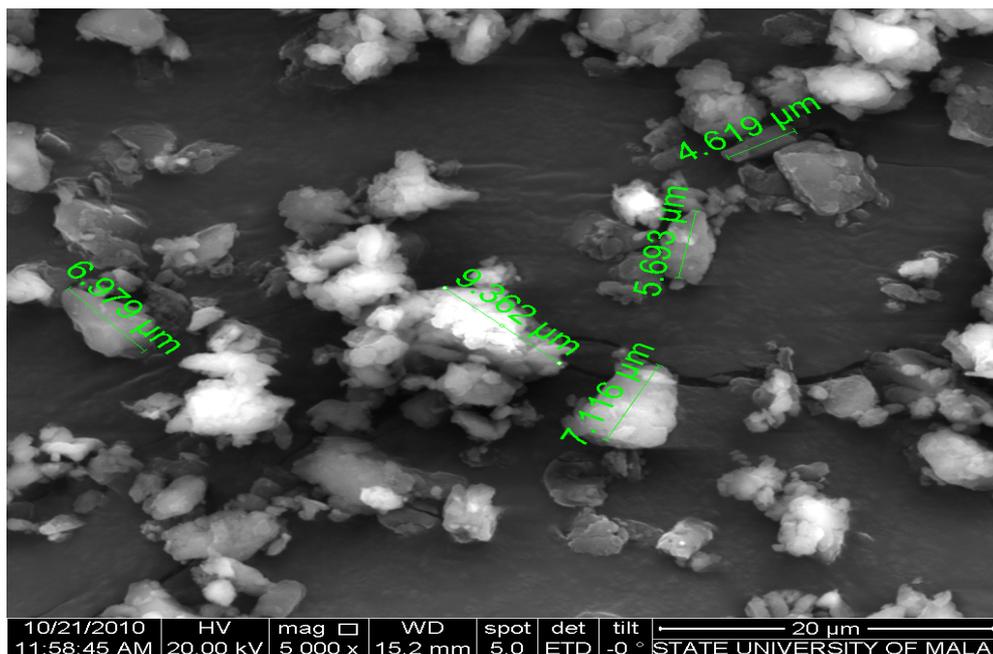


Figure 1. Scanning electron microscopy of coal dust (magnification 5000 x)
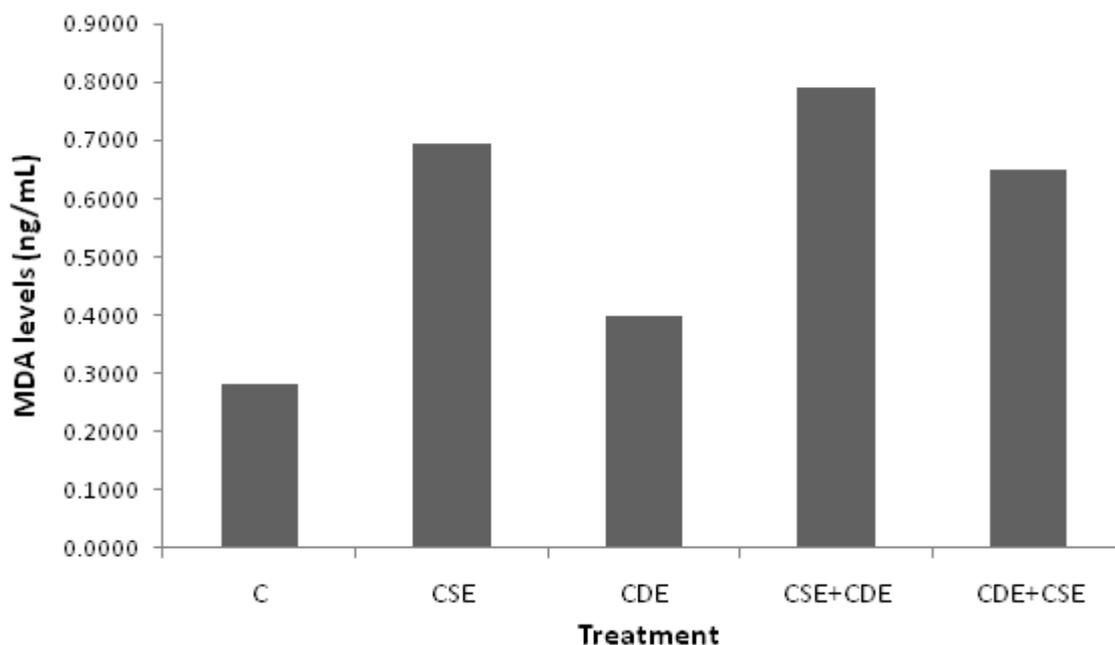
Figure 2. Mean MDA concentrations in the treatment groups.
C=control; CSE= cigarette smoke exposure; CDE= coal dust exposure; CSE+CDE= cigarette smoke exposure followed by exposure to coal dust; CDE + CSE= coal dust exposure  followed by cigarette smoke exposure

**Statistical analysis**

The collected data were tested for normality of distribution and variance. Normally distributed data of identical variance were analyzed using Anova. Significant differences between intervention groups ($p<0.05$) were followed up by multiple comparison test. Non-normally distributed data of differing variances were tested by non-parametric tests.

**RESULTS**

The technique for preparation of coal dust, as stated in the Methods section, produced coal dust particles with a maximum diameter of <10 μm, as shown in the scanning electron microscopy results in Figure 1. The coal dust particles of <10 μm had a variety of shapes and tended to form aggregates. X-ray fluorescence yielded information on the mineral content, comprising iron (Fe) 36.9%; silicon (Si) 17.9%; molybdenum (Mo) 15%; aluminum (Al) 10%; calcium (Ca) 8.67%;

sulphur (S) 4.7%; and titanium (Ti) 3.65%. Other minerals of <1% included vanadium (V), chromium (Cr), manganese (Mn), nickel (Ni), copper (Cu), and yterbium (Yb).

Mean MDA levels of the treatment groups are shown in Figure 2. The collected data were not suitable for parametric tests, and were therefore analyzed using non-parametric tests. In the single exposure groups for cigarette smoke and coal dust, and the combined exposures to cigarette smoke, followed by coal dust, or coal dust exposure plus cigarette smoke, Kruskal Wallis testing found significantly different increases in MDA levels between treatment groups (p=0.000). Futhermore, using the Mann Whitney test, significant differences were found between C vs CSE (p=0.009); C vs CDE + CSE (p=0.009); C vs CSE + CDE (p=0.009); CDE vs CSE + CDE (p=0.028). No significant differences were found between C vs CDE (p=0.117); CDE vs CDE + CSE (p=0.113); CSE vs CDE + CSE (p=0.463); CSE vs CSE + CDE (p=0.347); and CDE + CSE vs CSE + CDE (p=0.116).
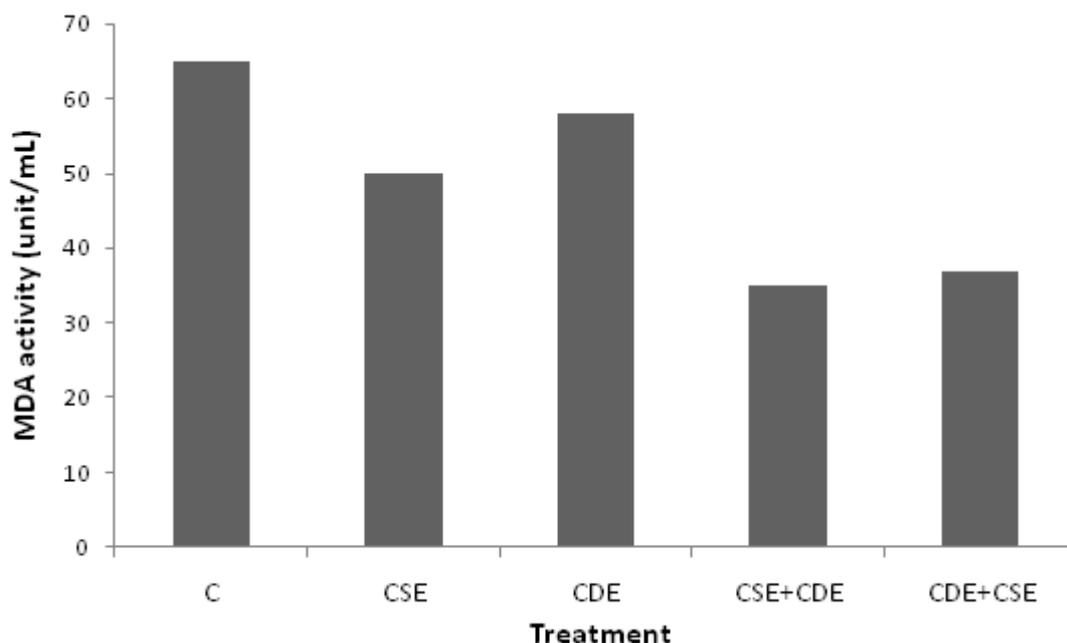
Figure 3. SOD activity in the treatment groups.
C=control; CSE= cigarette smoke exposure; CDE= coal dust exposure; CSE+CDE= cigarette smoke exposure follwed by exposure to coal dust; CDE + CSE= coal dust exposure follwed by cigarette smoke exposure
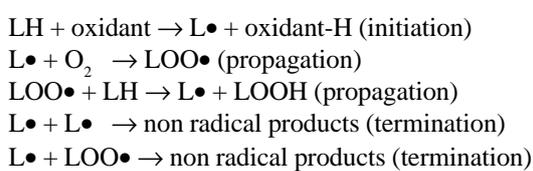
Mean SOD activities in the treatment groups are shown in Figure 3. The collected data were not suitable for parametric tests, and were therefore analyzed using non-parametric tests. In the single exposure groups for cigarette smoke and coal dust, and the combined exposures to cigarette smoke, followed by coal dust, or coal dust exposure plus cigarette smoke, Kruskal Wallis testing found significantly different decreases in SOD activity between treatment groups (p=0.000). The Mann Whitney test found significant differences between C vs CSE (p=0.009); C vs CDE (p=0.028); C vs CSE + CDE (p=0.009); C vs CDE + CSE (p=0.009); CDE vs CSE + CDE (p=0.009); CDE vs CDE + CSE (p=0.009); CSE vs CSE + CDE (p=0.0059); CSE vs CDE + CSE (p=0.016). No significant differences were found between CSE vs CDE (p=0.117); CDE + CSE vs CSE + CDE (p=0.917).

## DISCUSSION

The technique for preparation of coal dust produced coal dust particles with a maximum diameter of <10 mm, having a variety of shapes and tending to form aggregates. This aggregative tendency is presumably due to attraction between charged atoms of the coal dust particles, in the form of cations or oxo-ions. The diameter of the coal dust particles used in this study was less than the diameter of dust partilces used in the study by Pinho et al.,[7] but larger than in the study by Ghanem et al.[14] and Armutcu et al.[9] The unique physical characteristics (shape, size, surface area) and the metal constituents of these particles are major predictors for vascular oxidative stress. Nanoparticle are an important constituent part of PM10. Nanoparticle are closely associated with respiratory, cardiovascular diseases and even the death rate

due to their nanosize effects, high specific surface area and complicated chemical composition allowing redox reaction take place.[15-17]

The main finding in this study is that exposure to cigarette smoke and coal dust triggers systemic oxidative stress. This is consistent with the findings of Brook et al.[18] Malondialdehyde is a lipid peroxidation product involving a three-stage reaction, i.e. initiation, propagation, and termination. The overall reaction is:

$LH + oxidant \rightarrow L\bullet + oxidant\text{-}H$ (initiation)
$L\bullet + O_2 \rightarrow LOO\bullet$ (propagation)
$LOO\bullet + LH \rightarrow L\bullet + LOOH$ (propagation)
$L\bullet + L\bullet \rightarrow non\ radical\ products$ (termination)
$L\bullet + LOO\bullet \rightarrow non\ radical\ products$ (termination)

For single exposures, significant increases in blood MDA levels versus controls were found in the cigarette smoke exposure group. This finding is similar to the study by Murarescu et al., where increased serum MDA levels were found in rats exposed to cigarette smoke.[12] Coal dust exposure at a dosage of 12.5 mg/m$^3$ for 14 days failed to significantly increase blood MDA levels, compared with the controls. The results of the present study were at variance with the study of Armutcu et al., which demonstrated increased serum MDA levels in rats exposed to coal dust for 14 days (24 hours per day in underground mines).[9] This discrepancy in results is presumably due to the prolonged daily exposures in the study by Armutcu et al.

The underlying reason for the different effects of single cigarette smoke exposure (CSE) and single coal dust expsosure (CDE) is the gaseous form of the active substances in cigarette smoke, leading to diffusion directly of the free radicals to all parts of the lungs. The active substances can also diffuse via the alveolar membrane into the blood stream, where they immediately initiate lipid peroxidation reactions. This is in contrast with coal dust exposure, where the coal dust is initially phagocytosed to produce free radicals.

Phagocytosis is a cellular process requiring molecular and cellular communication, thus involving more time for initiating lipid peroxidation reactions. Details steps of phagocytosis consist of recognition, response, and removal.[19] Although coal dust contains catalytic minerals for redox reactions, such as Fe and Ti, these reactions also require the presence of $H_2O_2$ for hydroxyl radical formation.

For the combined exposures, all exposure groups had significantly increased blood MDA levels, compared to the control group, although there was no difference between CSE + CDE and CDE + CSE. The free radicals in the gaseous phase, the products of coal dust phagocytosis, and the redox catalyzation products will initiate lipid peroxidation reactions, although it cannot be determined which of these was the most dominant. There is a variety of reactive compounds produced by coal dust, including $\bullet O_2^-$, $H_2O_2$, and NO. Cigarette smoke contains $\bullet O_2^-$ and NO.[4] One of the catalytic redox reactions is the Fenton or Haber-Weiss reaction:[8]

$Ti(III) + H_2O_2 \rightarrow Ti(IV) + OH + \bullet OH$

In comparing the single exposures with the combined exposures, a significant increase was found for CDE vs CSE + CDE. This is due to the diffusive capacity of gaseous reactive compounds derived from cigarette smoke, leading to their appearance in the blood, to be augmented with reactive compounds from coal dust. In contrast, for CDE vs CDE + CSE; CSE vs CDE + CSE; and CSE vs CSE + CDE, no significant differences were found. This was on the one hand presumably due to the high level of reactive compounds in cigarette smoke, and on the other hand to reactions between radicals neutralizing one another (termination reactions).

Superoxide dismutase is an enzymatic antioxidant that catalyzes the conversion of $\bullet O_2^-$ into $H_2O_2$. The function of SOD is to convert radicals into oxidants, or reduce the reactivity of radical molecules. The effect of

single cigarette smoke exposures on SOD was to significantly reduce SOD activity, in comparison to controls, which was also the case with coal dust exposure. This is presumably caused by the gaseous phase free radicals in cigarette smoke ($\bullet O_2^-$) triggering spontaneous dismutation of SOD in the blood, through the diffusion of cigarette smoke gases into the circulation. In the case of single exposures to coal dust, the reduced SOD activity was due to non-phagocytic mechanisms, in the form of reactivity of inorganic components, or to phagocytic mechanisms resulting in superoxide radical production. The effect of coal dust exposure on blood SOD activity in this study was contrary to the study by Armutcu et al.,[9] who found no significantly increased SOD activity even with exposure times of 24 hours per day. This difference in study results was due to a differences in exposure dose, namely 1.17 mg/m$^3$ in the study of Armutcu et al. and 12.5 mg/m$^3$ in the present study.

In the case of combined exposures, there was a significant decrease in SOD activity, as compared to controls or to single exposures. This may be because cigarette smoke and coal dust are sources of $\bullet O_2^-$ as the substrate of SOD. The point of interest is the nonsignificancy of single coal dust exposures in comparison with single cigarette smoke exposures, or combined exposures of both, indicating that the formation of $\bullet O_2^-$ in the circulation, as a result of cigarette smoke gases, has a similar effect as coal dust exposures. Beside that, tar phase of cigarettes smoke is an effective metal chelator wherein iron is chelated to produce tar-semiquinone + tar-Fe$^{2+}$, which can inhibit redox properties of metal.[20]

A limitation of this study is that we were unable to determine the types of the free radicals produced, in order to determine their respective roles in coal dust and cigarette smoke exposures. This is due to their considerably high reactivity, requiring electron paramagnetic resonance or electron spin resonance apparatus for identification of their types.

## CONCLUSIONS

Single exposure to cigarette smoke triggered significant increases in blood MDA levels and significant decreases ini serum SOD activity. Single coal dust exposure failed to trigger increases in blood MDA levels and only induced decreases in serum SOD activity. Combined exposures to cigarette smoke and coal dust triggered significant increases in blood MDA levels and significant decreases in serum SOD activity.

## ACKNOWLEDGEMENT

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