The effects of combined particulate matter 10 coal dust exposure and high-cholesterol diet on lipid profiles, endothelial damage, and hematopoietic stem cells in rats

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Key Words
Atherosclerosis; Coal dust; Endothelial damage; Inhalation toxicology; Lipid profile

Abstract
Objective: This study aimed to investigate the effect of combined particulate matter 10 (PM10) coal dust exposure and high-cholesterol diet on lipid profiles, circulating endothelial cells (CECs), and hematopoietic stem cells (HSCs) in rats.

Methods: Thirty male Wistar rats were randomly divided into six groups. Rats were fed a normal diet (non-exposure group), a high-cholesterol diet for 8 weeks (HCD control group), or a high-cholesterol diet concomitantly exposed to 12.5 mg/m3 of PM10 coal dust an hour daily for 5, 6, 7 or 8 weeks. Rats were sacrificed at the end of experiment, and then blood samples were collected. Lipid profiles were evaluated by colorimetric methods. CECs and HSCs were determined by flow-cytometric methods. Intergroup comparisons were performed by ANOVA.

Results: High-cholesterol diet significantly increased total cholesterol (TC), triglyceride, low-density lipoprotein-cholesterol (LDL-c) level, TC/high density lipoprotein-cholesterol (HDL-c) ratio, atherogenic index, and decreased HDL-c level compared to non-exposure group. Combined coal dust exposure and high-cholesterol diet significantly decreased TC, LDL-c level, TC/HDL-c ratio, atherogenic index, and increased HDL-c level relative to the HCD controls. Triglyceride level of the group with 6-week treatment was significantly higher compared to the non-exposure group achieving near-HCD control group levels. The HDL-c/LDL-c ratio, CECs, and HSCs were not significantly different among groups.

Conclusion: Concomitant coal dust exposure and high-cholesterol diet reduced atherogenic index due to decreased TC and LDL-c, and elevated HDL-c levels.

INTRODUCTION
Lipids have important roles in pathological conditions. Elevation in serum lipid levels, namely hyperlipidemia, can occur due to increased biosynthesis and/or decreased elimination of lipids. Hyperlipidemia is a traditional risk factor for cardiovascular disease (CVD), the leading cause of death in almost all countries [1]. Deposition of cholesterol, triglyceride, calcium, and other substances within the vascular system is the hallmark of atherosclerosis [2]. Epidemiological studies clearly showed that particulate matter inhalation increases morbidity and mortality of CVD [3]. In the population residing near the Appalachian coal mining areas (United States), there is increasing rates of CVD [4]. Several studies showed that coal dust produces free radicals [5] and increases oxidative stress in rats [6, 7] and humans [8, 9]. These induce oxidative modification of lipids leading to the development of atherosclerosis. This finding indicates that coal dust was an established risk factor of CVD, but whether coal dust exposure can accelerate hyperlipidemia in rats fed high-cholesterol diet (HCD) is still unknown.

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In arteries, endothelial cells are the first line of defense against damaging effect of hyperlipidemia [10]. Hyperlipidemia can induce abnormal endothelial cells function and increase circulating (detached) endothelial cells (CECs) [11]. To decrease endothelial cells damage, hematopoietic stem cells (HSCs) differentiate into endothelial cells [12]. The transplantation of allogeneic HSCs significantly decreased CECs numbers [13]. There is no study investigating the involvement of CECs and HSCs on hyperlipidemic rats exposed to coal dust.

In this study, we investigated the changes in lipid profiles, endothelial damage, and HSCs in male Wistar rats exposed to particulate matter 10 (PM$_{10}$) coal dust with concomitant HCD. We hypothesized that concomitant PM$_{10}$ coal dust exposure and HCD would increase atherogenic index due to increased level of cholesterol, triglyceride and low-density lipoprotein-cholesterol (LDL-ct), and decreased level of high-density lipoprotein-cholesterol (HDL-ct), and also induce detrimental effect on endothelial cells and damage the HSCs pool.

**MATERIALS AND METHODS**

**Animals**

Thirty male Wistar rats weighing 110-145 g purchased from Central Animal House of Bandung were housed in an air-conditioned room at 24 ± 2°C and 65-70% relative humidity with a 12 h light-dark cycle.

**Ethics**

Animal care and experimental procedures were approved by the institutional animal ethics committee of University of Lambung Mangkurat, Banjarmasin.

**Coal dust preparation**

Coals were obtained from coal mining area in South Kalimantan, Indonesia. Two kilograms of gross coals were pulverized by Ball Mill, Ring Mill, and Raymond Mill in Carsurin Coal Laboratories of Banjarmasin. Coal dust particles then were filtered by Mesh MicroSieve (BioDesign, New York, NY, USA) to obtain particles with diameter less than 10 μm (PM$_{10}$). Specimens of coal dust then were kept in ‘Tissue and Specimen Bank of Banjarmasin’ with reference code 2012-2-CD. Morphology and diameter size of particles were characterized by a scanning electron microscope (SEM). Inorganic composition was analyzed by X-ray fluorescence. The type, size, and crystalline percentage of coal dust were also analyzed by X-ray diffraction. All these instruments were available at Physic and Central Laboratory, Faculty of Mathematics and Natural Science, University of Malang.

Coal dust particles were less than 10 μm which was confirmed by SEM in one dimension were considered as PM$_{10}$ coal dust. Coal dust particles consisted of single particles and aggregate particles. Inorganic composition of coal dust were iron (29.3 ± 0.1%), silicon (29 ± 0.2%), calcium (12 ± 0.07%), aluminum (10 ± 0.2%), titanium (6.31 ± 0.19%), phosphorus (5.9 ± 0.04%), potassium (4.5 ± 0.06%), barium (1 ± 0.09%), and several inorganic minerals less than 1% including europium (0.7 ± 0%), chromium (0.48 ± 0.04%), nickel (0.41 ± 0%), copper (0.34 ± 0.02%), zinc (0.22 ± 0.03%), vanadium (0.2 ± 0.02%), and manganese (0.15 ± 0.09%). X-ray diffraction showed 36.3% of crystalline with 177 nm crystal size consisting of illite (potassium aluminum silicate hydroxide hydrate), viseite (calcium aluminum phosphate silicate hydroxide), and cromstedtite (iron silicate hydroxide) [14].

**Coal dust exposure**

Thirty male Wistar rats were randomly divided into six groups. Rats were fed a normal diet (non-exposure group), a HCD for 8 weeks (HCD control group), or a HCD with concomitant exposure to 12.5 mg/m$^3$ of coal dust an hour daily for 5, 6, 7 or 8 weeks, respectively. The concentration of coal dust was determined according to occupational exposure in upper ground coal mining area in South Kalimantan, Indonesia [15, 16]. In Indonesia coal mining areas, miners start day shift in the morning then take a midday break for lunch. To mimic this real condition, coal dust exposure was performed prior to HCD.

Coal dust exposure was done using exposure chamber designed and available in Pharmacology Laboratory, Faculty of Medicine, Brawijaya University. The principal work of the equipment is to provide an ambient resuspended PM$_{10}$ coal dust which can be inhaled by rats and resembles the environmental airstream. Chamber size was 0.5 m$^3$ and flowed by a 1.5-2 l/min airstream. To prevent hypoxia and discomfort, we also provide oxygen supply in the chamber. Non-exposure group and HCD control group were exposed to filtered air in laboratory [14].

**Diets**

Diets were made following the American Institute of Nutrition (AIN) recommendations. Animals were given water *ad libitum* during the experimental period. The composition of normal diet was 66% comfeed PAR-s, 33% wheat powder, and water. The composition of HCD was 53% comfeed PAR-s, 26.5% wheat powder, 0.1% sheep oil, 0.0013% cholic acid, and 3.22% pig oil, according to previous study with modification [17].

**Serum lipid profiles analysis**

At the end of the experiment, rats were anesthetized with diethyl ether, and then blood samples were obtained by cardiac puncture. The serum was separated by 4 min of centrifugation at 4000g and 4°C. All
samples were stored at -80°C until analyzed. Concentrations of cholesterol, triglyceride, and HDL-c in plasma were determined by enzymatic colorimetric methods using commercial kits (Spinreact, Girona, Spain). LDL-c level was calculated according to the Friedewald equation [18]:

\[ \text{LDL-c} = \text{TC} - \text{HDL-c} - \text{TG} / 5 \]

The atherogenic index (AI) was defined according to the formula given below and calculated for all experimental groups:

\[ \text{AI} = (\text{TC} - \text{HDL-c}) / \text{HDL-c} \]

### Measurement of hematopoietic stem cells

HSCs were isolated from blood as described elsewhere [19] with modification. Briefly, 3 ml of EDTA-blood was obtained by cardiac puncture, stored at 4-10°C, and processed within 6 h after collection. Mononuclear cells were isolated by density-gradient centrifugation using 1.077 g/ml Ficoll-Paque (Sigma-Aldrich, St. Louis, MO, USA). Isolated cells were washed twice with PBS and re-suspended in PBS supplemented with 2% of fetal bovine serum. CD34+ cells were evaluated by immunostaining with PE conjugated monoclonal antibody (BioLegend, London, UK) and detected by flow cytometry (BD FACSCalibur™ Flow Cytometer; BD Biosciences, San Jose, CA, USA).

### Measurement of circulating endothelial cells

Briefly, 3 ml of EDTA-blood was obtained by cardiac puncture, stored at 4-10°C, and processed within 6 h after collection. Mononuclear cells were isolated by density-gradient centrifugation using 1.077 g/ml Ficoll-Paque (Sigma-Aldrich). Isolated cells were washed twice with PBS and re-suspended in PBS supplemented with 0.5% of fetal bovine serum. CD146+ cells were evaluated by immunostaining with FITC-conjugated CD146 monoclonal antibody (BioLegend) and detected by flow cytometry.

### Statistical analysis

Data are presented as mean ± SD and the differences between groups were analyzed using One-way analysis of variance (ANOVA) with SPSS 15.0 software. Post hoc test was used if the ANOVA was significant. Probability values of P < 0.05 were considered statistically significant.

### RESULTS

#### Lipid profiles

Table 1 shows the lipid profiles in all groups. The level of TC, triglyceride, LDL-c, TC/HDL-c ratio and AI were significantly higher in HCD control group compared to non-exposure group (P < 0.05). The level of HDL-c was significantly lower in HCD control group compared to non-exposure group (P < 0.05). The HDL-c/LDL-c ratio was not significantly different between groups (P > 0.05). Combined coal dust exposure and HCD significantly decreased total cholesterol, LDL-c level, TC/HDL-c ratio, and AI, and also increased HDL-c level relative to HCD control group (P < 0.05). A 6-week coal dust exposure in rats fed HCD significantly increased triglyceride level as compared to that in non-exposure group (P < 0.05) achieving near-HCD control group levels.

<table>
<thead>
<tr>
<th></th>
<th>Non-exposure</th>
<th>High-cholesterol diet</th>
<th>12.5 mg/m² of PM₁₀ coal dust exposure + high-cholesterol diet</th>
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<tbody>
<tr>
<td></td>
<td>5 weeks</td>
<td>6 weeks</td>
<td>7 weeks</td>
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<td>8 weeks</td>
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<tr>
<td>TC (mg/dl)</td>
<td>59 ± 6.27</td>
<td>207.5 ± 19.9a</td>
<td>72.5 ± 14.36b</td>
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<td>57.75 ± 5.5b</td>
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<td>55.75 ± 2.87b</td>
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<td>56.5 ± 4.5b</td>
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<tr>
<td>Triglyceride (mg/dl)</td>
<td>86.5 ± 27.25</td>
<td>138.5 ± 12.17a</td>
<td>97 ± 48.51</td>
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<td>114.25 ± 32.66a</td>
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<td>47 ± 23.73</td>
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<td>104 ± 48.14</td>
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<td>HDL-c (mg/dl)</td>
<td>29.42 ± 2.33</td>
<td>21 ± 2.16a</td>
<td>34.17 ± 6.46b</td>
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<td>28 ± 2.86b</td>
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<td>28.27 ± 3.04b</td>
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<tr>
<td>LDL-c (mg/dl)</td>
<td>12.27 ± 6.52</td>
<td>158.75 ± 19.56a</td>
<td>18.92 ± 9.31b</td>
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<td>7.15 ± 5.71b</td>
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<td>11.57 ± 3.86b</td>
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<td>HDL-c/LDL-c</td>
<td>3.06 ± 1.69</td>
<td>0.92 ± 0.89</td>
<td>2.22 ± 1.35</td>
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<td>3.54 ± 1.75</td>
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<td>1.78 ± 0.59</td>
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<td>2.49 ± 0.9</td>
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<td>TC/HDL-c</td>
<td>2 ± 0.17</td>
<td>9.98 ± 1.51a</td>
<td>2.11 ± 0.09b</td>
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<td>2.07 ± 0.27b</td>
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<td>1.94 ± 1.17b</td>
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<td>2 ± 0.31b</td>
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<tr>
<td>AI (units)</td>
<td>1 ± 0.17</td>
<td>8.98 ± 1.51a</td>
<td>1.11 ± 0.09b</td>
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Values are presented as mean ± SD. *P < 0.05 in comparison with; *non-exposure group, *rats fed high-cholesterol diet, *rats exposed to coal dust with concomitant high-cholesterol diet for 5 weeks, *rats exposed to coal dust with concomitant high-cholesterol diet for 6 weeks, *rats exposed to coal dust with concomitant high-cholesterol diet for 7 weeks. HDL-c: high-density lipoprotein-cholesterol; LDL-c: low-density lipoprotein-cholesterol; TC: total cholesterol; AI: atherogenic index.

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Hematopoietic stem cells
The amount of HSCs was significantly higher in rats fed HCD with concomitantly exposed to coal dust for 5 weeks and 8 weeks compared to 6-week exposure (P < 0.05) as shown in Table 2.

Circulating endothelial cells
The amount of CECs was not significantly different between groups (P > 0.05) as shown in Table 2.

**DISCUSSION**
Lowering total cholesterol, triglyceride, and LDL-c level is a strategy to decrease the atherosclerosis progression. In the present study, coal dust exposure resulted in ∼65-73% lower total cholesterol compared to rats fed HCD. This may be due to the action of inorganic components of coal dust in the reverse cholesterol transport pathways. Several inorganic components of coal dust which may have taken part in decreasing cholesterol level are silicon, chromium, vanadium, copper and manganese. A previous study demonstrated that elevated silicon ingestion impedes cholesterol-induced atherogenesis in rabbits [20]. Chromium also induces cholesterol loss *in vitro* [21] and *in vivo* [22, 23]. In addition, vanadium effectively reduces hypercholesterolemia in diabetic rats [24]. Besides, the dietary intakes of copper and manganese have been reported to play an important role in controlling lipids in Korean adults [25].

Combined 6-week PM₁₀ coal dust exposure and HCD significantly increased triglyceride level compared to non-exposure group achieving near-HCD control group levels. On the contrary, combined 7-week PM₁₀ coal dust exposure and HCD significantly decreased triglyceride level compared to HCD control group, restored to normal level. Silva et al [26] found that iron overload can increase triglyceride in rats. Another study reported that iron loading impairs lipoprotein lipase and promotes hypertriglyceridemia [27]. We found that iron is the highest inorganic component of coal dust used in this study; this may have induced the elevated triglyceride level in the 6-week treatment group.

Combined PM₁₀ coal dust exposure and HCD restored HDL-c to normal level. There was also decreased LDL-c level and TC/HDL-c ratio. HDL-c has anti-atherosclerotic effects due to its ability to reverse cholesterol transport [28, 29]. The transfer of cholesteryl esters (CE) from HDL-c to apoB-containing lipoproteins is facilitated by cholesteryl ester transfer protein (CETP), and then it is cleared from the circulation by the liver [30]. This indicates that coal dust may increase the activity of cholesteryl esters transfer due to increased HDL-c level. However, in CVR, reduced level of TC/HDL-c ratio is a marker of decreased morbidity and mortality [31].

Athero-protective properties of HDL-c may be related with its role in maintaining endothelial function [31]. This study showed that, although not statistically significant, HCD increased endothelial cells damage. Coal dust exposure also tended to decrease endothelial cells damage, except in 6-week treatment. Subsequently, the endothelial cells viability determines the release and mobilization of HSCs from bone marrow. In the niches, HSCs are in a quiescent state to maintain HSCs pool and to be alert to respond to the signals of blood cells or HSCs pool imbalance caused by either intrinsic or extrinsic stimuli [12]. This study indicated that concomitant coal dust exposure and HCD in rats did not damage the HSCs pool.

In conclusion, it was previously reported that coal dust exposure or HCD are associated with enhanced cardiovascular risk. Here we investigated the effects of combined PM₁₀ coal dust exposure and HCD to determine whether coal dust exposure can accelerate hyperlipidemia in rats fed HCD. This study showed there was decreased AI due to reduced level of cholesterol and LDL-c, and also elevated level of HDL-c.

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**COMPETING INTERESTS**
The authors declare that there are no conflicts of interest.
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