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Original Article

Subchronic inhalation of coal dust particulate matter 10 changes bone mesostructure, mineral element levels and turnover markers in rats

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Key Words
Inhalation; Femur; Rats; Toxicity; Trabecular bone

Abstract

Objective: This study aimed to elucidate whether inhalation particulate matter 10 (PM_{10}) of coal dust changes mesostructure, bone mineral elements, and turnover markers of rats.

Methods: Thirty-two male Wistar rats were randomly divided into four groups; one non-inhaled group and three coal dust exposed groups (concentration 6.25, 12.5, and 25 $mg/m^3/h/day$ for 28 days). Femur mesostructure were analyzed by scanning electron microscope. Bone mineral elements was assayed by X-ray fluorescence. Osteocalcin and C-telopeptide of type I collagen were analyzed by ELISA. ANOVA test was used to analyze the difference level of all markers.

Results: Mesostructure of non-inhaled rats presented rod like trabeculae with honey comb appearance and minimal hole. Disregular integrity of trabeculae and reduction of trabecular integrity, increasing porocity were found at coal dust exposed groups. The level of osteocalcin and C-telopeptide of type I collagen were significantly lower in coal dust exposed groups compared to control group. The levels of phosphorus and nickel were significantly lower in coal dust exposed groups compared to control group.

Conclusion: The present study reported that sub-chronic inhalation of coal dust PM_{10} changes bone mesostructure, phosporus and nickel levels in bone, and bone turnover markers of rats' femur.

INTRODUCTION

Bone remodeling is the renewal process of bone as a dynamic tissue throughout life in a process whereby osteoclasts resorb older bone and osteoblasts synthesize new bone [1]. Bone quality was also determined by structural and material properties [2, 3]. Atomic minerals are the smallest unit components of bone hydroxyapatite crystal. Substitution replaces a comparable atom due to similarities in their radii and charges to hydroxyapatite crystal; substitution potentially changes hydroxyapatite crystal, the behavior of bone cell, and mesostructure of bone. Finally, these changes will determine the bone quality [4].

Inhaling coal dust in occupational and atmospheric setting has contributed significantly to the development of several respiratory disorders and cardiovascular disease [5, 6], but the effect on bone is not clear. Our preliminary *in vivo* study concluded that coal dust decrease osteoblast population and increase osteoclast population in rats exposed to coal dust [7]. Coal mine dust is constituted from carbon, hydrogen, oxygen, nitrogen, inorganic minerals, quartz, and trace metals such as cadmium, iron, boron, copper, nickel, antimony, zinc, and lead [8]. Ren *et al* [9] showed that inhibition crystal growth will exist due to zinc substitution on hydroxyapatite crystal. Alfven *et al* [10] concluded that low inhalation of cadmium increase osteoporosis risk. Supplementation of nickel alone or combination of nickel and zinc also affect the structure of compact bone [11].

So far, no detailed information exists regarding to the asssociation of coal dust exposure to mesostructure, bone turnover markers, and bone mineral elements. We hypothesized that inhalation of coal dust could change bone turnover markers and bone mineral element levels. Furthermore, these mechanisms would influence bone mesostructure. Thus, the main aim of this study was to verify whether inhaled coal dust significantly change bone mesostructure due to decrease or increase of bone mineral elements and bone turnover markers.

MATERIALS AND METHODS

Animal

Adult male Wistar albino rats weighing 170-200 gram were used for the present *in vivo* investigation. They were housed in a clean wire and plastic cage and maintained under standard laboratory conditions of 25 ± 2°C temperature with a 12/12 h dark/light cycle. The animals were acclimatized to laboratory conditions for two weeks prior to the experiment and fed a standard pellet diet and received water *ad libitum*. All experimental animal procedures were described according to the Guide for the Care and Use of Laboratory Animals.

Coal dust preparation

Sub-bituminous gross coal was obtained from coal mining in South Kalimantan. One kilogram of coal was pulverized using a Ball Mill, Ring Mill and Raymond Mill in Carsurin Coal Laboratories of Banjarmasin resulting coal dust with a diameter of particle less than 75 μm (PM_{75}). To obtain particulate matter 10, PM_{75} was then filtrated by Mesh MicroSieve (BioDesign, USA) resulting a coal dust of less than 10 μm of diameter. Particulate matter 10 of coal dust was characterized by scanning electron microscope (SEM), X-ray fluorescence and X-ray diffraction at Physic and Central Laboratory, Faculty of Mathematics and Natural Science, University of Malang.

Coal dust aerosolization

A total of 32 male Wistar rats were randomly divided into four groups including one non-exposed group as sham and three coal dust exposed groups. The concentration of coal dust exposures were 6.25, 12.5, and 25 mg/m^3 one hour/day for 28 days based on previous studies [12, 13]. This concentration is based on coal dust concentration in upperground coal mining. Coal dust aerosolization was administrated using a special chamber that was designed and available in the Pharmacology Laboratory, Medical Faculty, Brawijaya University of Malang, as shown in Fig.1. The principal of this equipment is to provide a circulated ambient environment which contains coal dust to be inhaled by rat. Airstream in this chamber is 1.5-2 liters per minute that mimics an environmental airstream. To maintain oxygen supply, the chamber was connected to a pure oxygen tank.

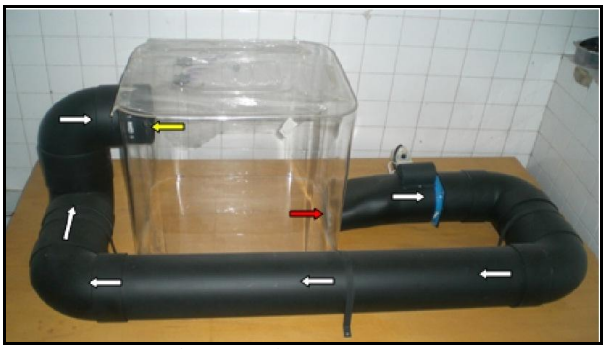

Figure 1. Design of chamber for coal dust exposure. The principal work of this equipment was to circulate (white arrow) an ambient airstream which contain coal dust in certain concentration. We put weighed coal dust in bottom hole (red arrow) of black pipe; then the coal dust will circulate and enter the chamber again via upper hole (yellow arrow). This airstream will inhaled by rats in plastic chamber. To avoid discomfort, this chamber is also supplied by external oxygen and placed in an air conditioned room.

Sample preparation

At the end of the treatment, the animals were anesthetized by ether; both femurs were collected and latter rinsed with physiological saline. All samples were stored in glutaraldehyde until analyzed. In addition, serum was also obtained by cardiac puncture for bone turnover marker analysis.

Mesostructure analysis

Mesostructure analysis was evaluated by SEM. For SEM evaluation, femurs from all groups were cut vertically from the proximal metaphysis area. Then the femur bones were fixed with phosphate formalin buffer, dehydrated with graded concentration of ethanol and coated with gold and palladium. The processed bones were then analyzed at 20 kV accelerating voltage by a SEM (FEI Inspect^{TM} S50). All procedures were done at Physic and Central Laboratory, Faculty of Mathematics and Natural Science University of Malang.

Bone turnover markers analysis

The serum bone formation markers osteocalcin was measured using Rat Osteocalcin/Bone Gla Protein OT/BGP ELISA kits from NovaTeinBio, Inc (Cambridge, MA, USA). The serum bone resorption marker C-telopeptide of type I collagen kit was purchased from NovaTeinBio, Inc (Cambridge, MA, USA).

Bone mineral element analysis

Bone mineral element analysis was evaluted by X-Ray Fluorescence (XRF). For XRF analysis, the femur bones inserted in bone tube, then put in proper place in

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equipment. The processed bones were then analyzed at 20 kV accelerating voltage by a XRF (PANalytical MiniPAL 4). All procedures was done at Physic and Central Laboratory, Faculty of Mathematic and Natural Science, University of Malang.

Ethics

This research has been approved by the research ethics committee of the Faculty of Medicine, University of Lambung Mangkurat, Banjarmasin, Indonesia.

Statistical analysis

Data are presented as mean ± SD and differences between groups were analyzed using ANOVA test of the SPSS 15.0 software; $P < 0.05$ was considered statistically significant.

RESULTS

Characterization of PM_{10} coal dust

The highest diameter of particle is less than 10 µm (in one dimension) which confirms coal dust particulate matter 10 (PM_{10}); we found also particulate matter in nanosize. The morphology of PM_{10} coal dust was small agglomerated of particle linked together formed larger aggregate particle [13]. Crystal analysis using X-ray diffraction showed 36.3% crystalinity, consisted of illite (pottasium aluminum silicate hydroxide hydrate), viseite (calcium aluminum phosphate silicate hydroxyde), and cronstedtite (iron silicate hydroxyte). Crystal size of coal dust was 177 nm.

The composition of coal dust consists of organic (28.14%) and anorganic (71.86%) phase. Anorganic component of coal dust was characterized using X-ray fluorescence at the Physic and Central Laboratory, Faculty of Mathematics and Natural Science, State University of Malang. Inorganic composition (%) of coal dust were iron (29.3 ± 0.1), silicon (29 ± 0.2), calcium (12.00 ± 0.07), aluminum (10 ± 0.2), titanium (6.31 ± 0.19), phosphorus (5.9 ± 0.04), potassium (4.5 ± 0.06), and barium (1 ± 0.09) and several inorganic minerals less than < 1% including europium (0.7 ± 0.001), chromium (0.48 ± 0.04), nickel (0.41 ± 0.001), copper (0.34 ± 0.02), zinc (0.22 ± 0.03), vanadium (0.2 ± 0.02), and manganese (0.15 ± 0.09).

Effect of PM_{10} coal dust on mesostructure

Mesostructure of non-inhaled rats presented rod like trabecules with honey comb appearance and minimal holes as shown in Fig.2A. Mesostructure of coal dust exposed groups at concentrations of 6.25 and 12.5 mg/m^3 were similar to control group but showed reduction of trabecular integrity and minimal hole as seen in Fig.2B and 2C. Mesostructure of coal dust exposed groups at 25 mg/m^3 concentration showed maximal reduction of trabecular integrity and massive hole to see in Fig.2D.

Effect of PM_{10} coal dust on bone turnover markers

After sub-chronic inhalation, the mean level of osteocalcin and C-telopeptide of type I collagen are shown in Table 1. The levels of osteocalcin and C-telopeptide of type I collagen resulted significantly lower in coal dust exposed groups compared to control group ($P < 0.05$).

Effect of PM_{10} coal dust on bone mineral elements

After sub-chronic inhalation, the mean level of phosphorus, calcium, iron, nickel and zinc are shown in Table 2. Briefly, phosphorus ($P = 0.023$) and nickel ($P = 0.023$) levels significantly decreased in coal dust exposed groups; however, the levels of calcium, iron and zinc did not change significantly ($P > 0.05$).

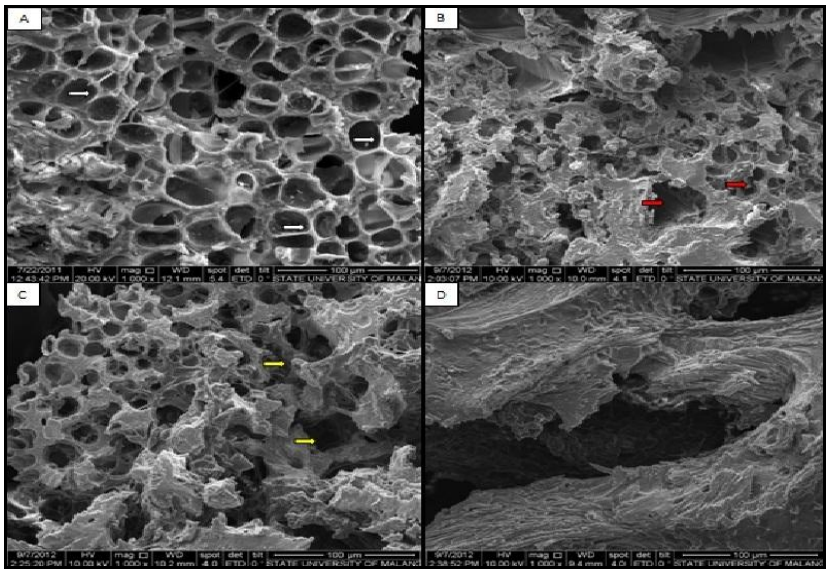

Figure 2.
Mesostructure of femur rat bone inhaled to coal dust.
(A) Mesostructure of non-inhaled rats showed rod like trabeculae (white arrow) with honey comb appearance; mesostructure of coal dust exposed groups at concentration 6.25 **(B)** and 12.5 mg/m^3 **(C)** showed reduction of trabeculae integrity and minimal holes (red and yellow arrow);
(D) mesostructure of coal dust exposed groups at concentration 25 mg/m^3 show maximal reduction of trabecular integrity and massive hole.

Table 1. Levels of bone turnover markers in coal dust exposed groups and control rats (ng/ml)

	Concentration of coal dust			
	0 mg/m^3 (Control)	**6.25 mg/m^3**	**12.5 mg/m^3**	**25 mg/m^3**
Osteocalcin	1.6946 ± 0.0875	1.0803 ± 0.1159[a]	1.0948 ± 0.1035[a]	1.0688 ± 0.0741[a]
C-Telopeptide	1.0175 ± 0.0975	0.558 ± 0.1258[a]	0.5211 ± 0.0517[a]	0.4947 ± 0.0767[a]

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

Table 2. Levels of bone mineral element in coal dust exposed groups and control rats (%)

	Concentration of coal dust			
	0 mg/m^3 (Control)	**6.25 mg/m^3**	**12.5 mg/m^3**	**25 mg/m^3**
Phosphorus	12.25 ± 0.17	6.15 ± 0.74[a]	7.84 ± 3.56[a]	10.88 ± 3.14[abc]
Calcium	79.90 ± 8.22	79.15 ± 0.52	80.63 ± 3.27	83.75 ± 1.34
Iron	5.4 ± 4.02	0.76 ± 0.1	0.88 ± 0.3	0.55 ± 0.22
Nickel	0.48 ± 0.63	0.1 ± 0.001[a]	0.13 ± 0.1[a]	0.1 ± 0.08[a]
Zinc	0.73 ± 0.39	1.2 ± 0.23	1.32 ± 0.45	0.72 ± 0.27

Values are presented as mean ± SD; [a]P < 0.05 in comparison with control group; [b]P < 0.05 in comparison with 6.25 mg/m^3 coal dust exposed group; [c]P<0.05; in comparison with 12.5 mg/m^3 coal dust exposed group.

DISCUSSION

Scanning electron microscopy showed clearly that the highest diameter of coal dust particles is less than 10 μm, at least one dimension, confirming as coal dust PM_{10}. The range of aerodynamic diameters is microparticles until nanoparticles. Size of crystal coal dust was 177 nm which demonstrated that small- or nano-agglomerated particles linked together and formed larger aggregate particles. The surface area of nanoparticles was higher compared to microparticles which determine their reactivity. In addition, single crystal coal dust potentiates to circulate systemically.

Our previous study showed that there is positive correlation between coal dust concentration and macrophage count indicating higher concentration of exposure induces higher deposited coal dust in lung. In the present study the highest anorganic component of coal dust was iron (29.3 ± 0.1%) which was also confirmed by a previous study [14]. This study does not include female animals because man are the dominant population among coal mining workers.

The main finding of this study is that coal dust changes mesostructure of trabecular bone, decreases bone turnover rate, and changes bone mineral element levels. As shown in Fig.2, the integrity of trabecular bone was disrupted in coal dust exposed groups compared to control group. In addition, the maximal reduction of trabeculae was found at highest concentration of coal dust compared to lower concentrations or control group. This finding was similar to a previous study which showed that the number of trabecules was reduced in oophorectomized rats [15].

C-telopeptides of type I collagen are products of collagen degradation and therefore reflects bone resorption; elevated C-telopeptide levels generally indicate accelerated bone turnover [2]. Osteocalcin, one of the very few molecules exclusively produced by osteoblasts, is a widely used marker for bone formation [16]. The levels of osteocalcin and C-telopeptide of type I collagen were significantly lowered in coal dust exposed groups compared to control. This finding indicated that sub-chronic inhalation of to coal dust decrease bone turnover and bone formation in rats. The mechanisms of decreasing bone turnover may involve decreased osteoclast activity due to increased phagocytic acivity of macrophages. A previous study showed that silica nanoparticles mediate an inhibitory effect on osteoclasts *in vitro* [17]. In addition, the mechanisms of decreasing bone formation may involve an apoptotic process of osteoblasts.

As given in Table 2, the phosphorus and nickel levels in coal dust exposed groups is significantly lower compared with controls. Vallet-Regi and Arcos [18] proposed the possibilities of substitution of several atomic minerals in bone structure hydroxyapatite crystal. The inorganic minerals in coal dust that induce substitution in metal group of hydroxyapatite crystal are calcium and pottasium. In addition, silicon, sulphur, chromium and vanadium induce substitution of phosphorus group in hydroxyapatite crystal. Besides, inducing osteoblast apoptosis may be another pathway of anorganic components of coal dust leading to increased porocity and decreased integrity of trabecular bone. Iron and nickel in coal dust have toxic effect on the osteoblasts. Iron was shown to be toxic for bone cells to induce osteoporosis by disturbing mineralization [19, 20]. Nickel, in higher concentrations, is able to induce osteoblast apoptosis [21].

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The effect of aluminum on bone is still controversial. It was reported that aluminum inhibits bone formation through reduction of osteoblast activity, osteoid mineralization and matrix formation [22, 23]. Exposure to silicon as a single substance or as silicate in coal dust crystal increases phosphorus substitution in hydroxy-apatite crystal [24]. Manganese is a prominent element for mucopolysacchride metabolism that affects organic matrix formation; it can substitute calcium in bone matrix [25]. Potassium and barium also induce substitution of calcium in hydroxyapatite crystal [4]. Similarity of charge and atomic radius between Mn, Ca, Fe, Ba, and Na induce competitive substitution between these atoms. The calcium content in coal dust maintain the calcium proportion of hydroxyapatite crystal. The effects of europium and titanium in coal dust on bone tissue remain still unknown.

In conclusion, the present study showed that sub-chronic inhalation of PM_{10} coal dust changes bone mesostructure, bone phosphorus and nickel levels as well as bone turnover markers in femur of rats.

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