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ORIGINAL PAPER

Effect of Lead Nanoparticles Inhalation on Bone Calcium Sensing Receptor, Hydroxyapatite Crystal and Receptor Activator of Nuclear Factor-Kappa B in Rats

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ABSTRACT

This study aimed to investigate whether Pb nanoparticle exposure affects the bone calcium sensing receptor (CaSR), hydroxyapatite crystal, and receptor activator of nuclear factor-kappa B (RANK) in rats exposed to subchronic and chronic inhalation. Thirty two rats were randomly divided into eight groups. One group is a non-exposed group. While three groups were exposed to nanoparticles Pb at the following doses 6.25; 12.5; or 25 mg/m^3 an hour daily for 28 days. Another three groups were exposed to nanoparticles Pb at following doses 6.25; 12.5; and 25 mg/m^3 one hour daily for 6 months. The expression of trabecular CaSR was significantly decreased at the all doses subchronic exposure compared to the control group ($P < 0.05$). The CaSR expression significantly decreased in second and third doses subchronic exposure groups compared to the control groups ($P < 0.05$). With subchronic exposure, the crystal size was increased in second dose group and decreased in lowest and highest doses compared to the control (untreated) group. The crystal size and c-axis were decreased in all dose chronic exposures compared to the control (untreated) group. The expression of cortical RANK was significantly lower at the two lowest dose chronic exposures compared to the control group ($P < 0.05$). In conclusion, Pb nanoparticle inhibit hydroxyapatite crystal growth at least a part via down regulation of CaSR and RANK.

Key words: inhalation; crystal; calcium sensing receptor; trabecular; cortical.

1. INTRODUCTION

Lead (Pb) is a heavy metal that is more widespread than any other metal. Pb can enter the human body through the respiratory and digestive tract (1). Pb has been postulated to be stored in the three parts of the body, including blood, soft tissue, and bone. Pb which located in the bone with a half-life of 30-40 years. Pb follow the path of calcium to enter the cells of the body (2). Modeling using the crystal marker indicates that the incorporation of Pb into trabecular bone hydroxyapatite crystals will increase density and decrease in bone porosity. This indicates that exposure to Pb will improve the quality of trabecular bone. Pb competes with divalent ions when the absorption of nutrients. Some examples of the divalent ions are calcium and zinc. Pb competes with calcium, disrupt the regulation of cell metabolism by binding to receptors, second-messenger calcium, calcium transport blocking the calcium channels and calcium-sodium pump, as well as competing in the calcium-binding protein (3).

The calcium-sensing receptor (CaR) is a member of the superfamily of G protein-coupled receptors (4). The calcium-sensing receptor (CaR) is a seven transmembrane domain G-protein coupled receptor that was initially characterized as the sensor responsible for modulating parathormone and calcitonin release in response to changes in blood calcium levels (5). However, the CaR is more than just a calcium sensor; it is a fairly broad spectrum sensor of small cationic molecules, capable of transducing signals in response to changes in the concentration of heavy metals, including lead and cadmium (6), as well as cationic amino acids (7). Receptor activator of NF-kB, alternately identified as TNF-related activation-induced cytokine receptor (TRANCE-R) or osteoclast differentiation and activation receptor (ODAR) is the signaling receptor for RANKL. RANK has been designated TNFRSF11A, and is a type I 616 amino acid homo-trimerizing transmembrane

protein containing four extra-cellular cysteine-rich pseudorepeats. Trimerization is promoted by interaction with RANKL. RANK is strongly induced, especially in OC precursor cells, by M-CSF [8-11]. As far as we know, there is no study evaluating the effect Pb nanoparticle on bone CaSR and RANK expression, Therefore, this study aims to investigate whether Pb nanoparticle exposures affects the bone CaSR, hydroxyapatite crystal, and RANK in rats exposed to subchronic and chronic inhalation.

2. MATERIAL AND METHODS

Animals

Male Wistar albino rats, 16 weeks of age, weighing 175–200 grams, were used for this study. Thirty two rats were randomly divided into eight groups. One of the group is the non-exposed group. Three groups were exposed to Pb nanoparticles at doses of 6.25; 12.5; or 25 mg/m^3 an hour daily for 28 days. Another three groups were exposed to Pb nanoparticles at doses of 6.25; 12.5; and 25 mg/m^3 one hour daily for 6 months. Animals were kept in a clean wire cage and maintained under standard laboratory conditions with a temperature of 25 ± 3°C and dark/light cycle 12/12 h. Standard diet and water were provided ad *libitum*. Animals were acclimatized to laboratory conditions for two weeks prior to the experiment. Animal care and experimental procedures were approved by the institutional ethics committee of Faculty of Medicine, Padjadjaran University, Bandung, West Java, Indonesia.

Pb Nanoparticles exposure

Pb nanopowder was purchased from Intelligent Materials Pvt. Ltd (Nanoshell LLC, Wilmington, DE, US). The concentration of nanoparticles Pb exposure was determined according to occupational exposure in upper ground coal mining areas in South Kalimantan, Indonesia (12-15) and Turkey (16). The exposure chamber was designed and is available in the Laboratory of Pharmacology, Faculty of Medicine, Brawijaya University. The principal work of the chamber is to provide an ambient resuspended PM_{10} coal dust, which can be inhaled by rats. Chamber size was 0.5 m^3 and flowed by a 1.5-2 L/min airstream that resemble the environmental airstream. To prevent hypoxia and discomfort, we also provide oxygen supply was also provided in the chamber. The non-exposed group was exposed to filtered air in the laboratory.

Tissue sampling

At the end of the treatment, the animals were euthanized by anesthetizing with ether inhalation. The femur was collected, weighed, and washed with physiological saline. All femur samples were labeled and stored at - 80°C until analysis.

Labeling immunofluorescence staining of CaSR

Paraffin-embedded femur sections (10 µm thick) were immunostained according to the manufacturer's instructions (Santa Cruz Biotechnology, Dallas, TX, USA). Briefly, lung sections were deparaffinized in xylene and dehydrated through a graded ethanol series. Nonspecific protein bindings were blocked with 2% skim milk powder in PBS at RT for 20 min, followed by washing with PBS. Next, femur sections were incubated with rabbit anti-CaSR polyclonal (Santa Cruz Biotechnology) antibodies at specified dilutions for 1 h, followed by washing with PBS. The primary antibody bindings were then detected with goat anti-rabbit Rhodamine (Santa Cruz Biotechnology) and goat anti-mouse FITC (Santa Cruz Biotechnology) antibodies at specified dilutions for 1 h in the dark, followed by washing with PBS. All PBS washed steps consisted of three washes of 5 min each. The expressions of CaSR were analyzed by counting fluorescent intensity of cells (in arbitrary units; AU) in five random high-power (x400) microscope fields. The fluorescent images were recorded under a confocal laser scanning microscope (Olympus).

Analysis of bone hydroxyapatite crystal

Characterization of the X-ray diffraction results was performed by means of PANanalytical X'Pert PRO-MPD. Subsequent analysis was by means of the software programs High Score Plus, Crystal Maker and DDVIEW, complemented with the latest version of PDF2. Diffraction spectra were recorded at an angle of 2θ, from 200 to 60o, with a Cu-K a radiation source (wave length = 1.54056 Å, 40 mA, 40 kV) and step size of 0.05° (17).

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 statistical package for Windows. Only probability values of $p<0.05$ were considered statistically significant different and later subjected to Tukey's post hoc test.

3. RESULTS

Figure 1 presents the trabecular CaSR expression in subchronic and chronic exposure groups. The expression of trabecular CaSR was significantly decreased at the all doses subchronic exposure compared to the control group (P<0.05). There is no significant differences between these three doses of subchronic exposure (P>0.05). We found no significant difference in the expression of trabecular CaSR in chronic exposure groups compared to control group (P>0.05).

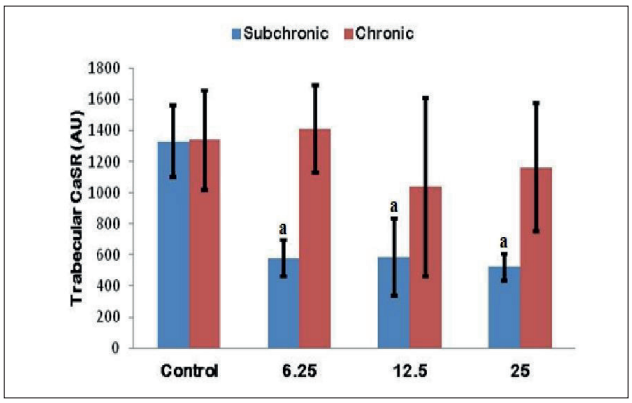

Figure 1. The expression of trabecular CaSR in each experimental group. Note: Values are presented as mean ± standard deviations; a p < 0.05 compared to the control group; CasR: calcium sensing receptor; AU: arbitrary units; dose exposure in mg/m3.

Figure 2 presents the expression of cortical CaSR in each experimental group. The CaSR expression significantly decreased in second and third doses subchronic exposure groups compared to the control groups (P<0.05). The expression of cortical CaSR was not significant differences between chronic exposure group (P>0.05).

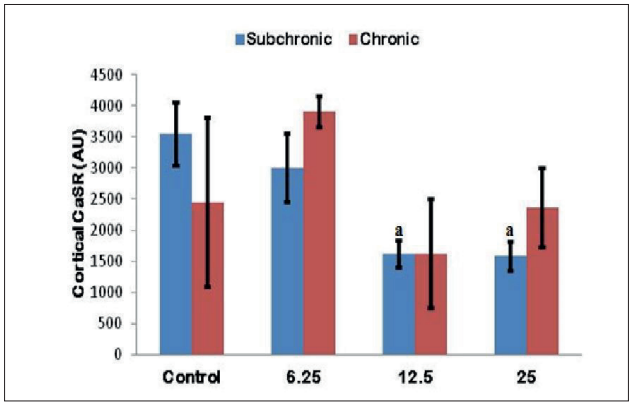

Figure 2. The expression of cortical CaSR in each experimental group. Note: Values are presented as mean ± standard deviations; a $p < 0.05$ compared to the control group; CasR: calcium sensing receptor; AU: arbitrary units; dose exposure in mg/m3.

Figure 3 presents the trabecular RANK expression in subchronic and chronic exposure groups. In subchronic exposure, the trabecular RANK expression was not significant difference between groups (P>0.05). We also found no significant difference of this expression in chronic exposure groups compared to control group (P>0.05).

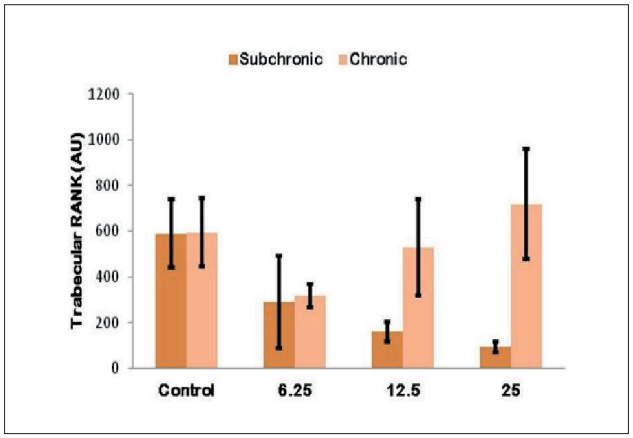

Figure 3. The expression of trabecular RANK in each experimental group. Note: Values are presented as mean ± standard deviations; RANK: receptor activator of nuclear factor-kappa B; AU: arbitrary units; dose exposure in mg/m3.

Figure 4 presents the cortical RANK expression in subchronic and chronic exposure groups. In subchronic exposure, the cortical RANK expression was not significant difference between groups (P>0.05). The expression of cortical RANK was significantly lower at the two lowest dose chronic exposures compared to the control group (P<0.05). Figure 5 present the crystal size and lattice parameters in subchronic exposure. The crystal size was increased in second dose group and decreased in lowest and highest doses compared to the control (untreated) group. The lattice parameters were different between groups. Figure 6 present the crystal size and lattice parameters in chronic exposure. The crystal size and c-axis were decreased at all doses compared to the control (untreated) group.

4. DISCUSSION

The CaSR is present in osteoblasts and osteoclasts and their precursors, as well as bone marrow-derived stromal cells, growth plate chondrocytes, cells of the monocyte–macrophage lineage and hematopoietic stem cells (18). The possible involvement of the CaSR in the etiology of osteoporosis, however, large-scale meta analysis of genome wide association data incorporating 150 candidate genes did not link the CaSR to bone mineral density or osteoporosis fracture risk (19). Stimulation through the CaSR leads to increased pro-

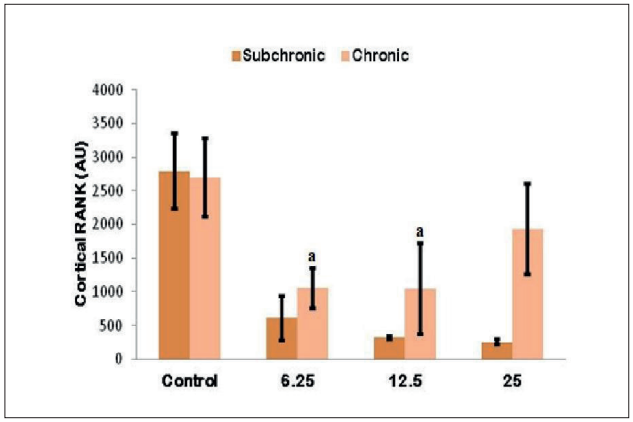

Figure 4. The expression of trabecular RANK in each experimental group. Note: Values are presented as mean ± standard deviations; a $p < 0.05$ compared to the control group; RANK: RANK: receptor activator of nuclear factor-kappa B; AU: arbitrary units; dose exposure in mg/m3.

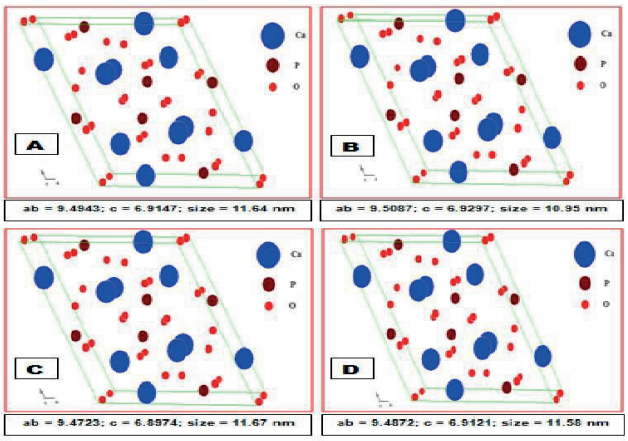

Figure 5. The crystal size and lattice parameters in subchronic exposure. The crystal size was increased (11.67 nm) in second dose group and decreased in the lowest (10.95 nm) and highest (11.58 nm) doses compared to the control (11.64 nm) group. The lattice parameters were different between groups. The c-axis was decreased in two highest doses of subchronic exposure compared with control.

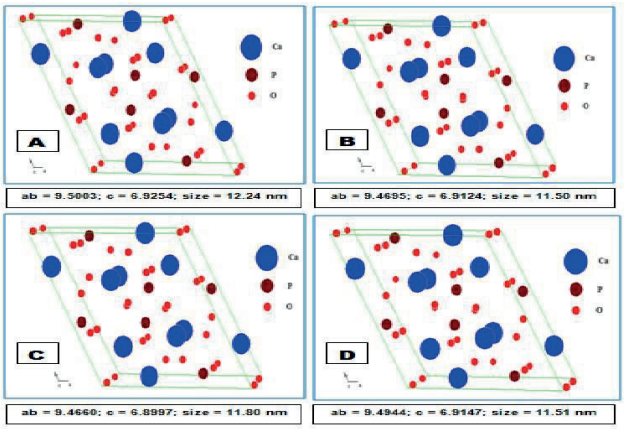

Figure 6. The crystal size and lattice parameters in chronic exposure. The crystal size was decreased in all dose exposure groups compared to the control group. The lattice parameters were different between groups. The c-axis was also decreased in all doses of chronic exposure compared with control.

duction of PTHrP a physiological regulator of bone formation having positive effects on osteoblast differentiation and survival (20, 21). Our study showed that the expression of trabecular CaSR was significantly decreased at the all doses subchronic exposure compared to the control group (P<0.05). There was no significant differences between these three doses of chronic exposure (P>0.05). In cortical femur, the CaSR expression significantly decreased in second and third doses subchronic exposure groups compared to the control groups (P<0.05). This finding indicated that Pb nanoparticles may have down-regulated CaSR expression in cortical or trabeculaer bone at critical match dose. This lag phase indicates the complexity of biological system (22, 23).

In this study, the crystal size was increased in second dose group and decreased in lowest and highest doses compared to the control group (subchronic exposure). This finding indicated that Pb nanoparticles modified the crystal size at critical match dose. The grow of mineral in hydroxyapatite crystal occurs under specific orientation in which the c-axis of the crystal is approximately parallel to the length axis of the collagen fiber (24). Specifically, the c-axis were decreased in two highest doses of subchronic exposure compared with control. With chronic exposure, the crystal size and c-axis were decreased in all doses compared to the control (untreated) group. We hypothesized that this crystal growth inhibition was, at least a part involved the down regulation of CaSR.

RANK is a homotrimeric transmembrane protein member of the TNF receptor superfamily (25). In subchronic and chronic exposure, the trabecular RANK expression was not significant difference between groups (P>0.05). The expression of cortical RANK was significantly lower at the two lowest dose chronic exposures compared to the control group (P<0.05). Our finding showed that Pb nanoparticles down-regulated the expression of cortical RANK. This finding indicates that Pb nanoparticles inhibits signal for osteoclast differentiation and bone resorption.

5. CONCLUSION

Pb nanoparticle inhibit hydroxyapatite crystal growth at least a part via down regulation of CaSR and RANK.

• Conflict of interest statement: The author(s) declare(s) that there is no conflict of interest regarding the publication of this paper.

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