Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ DERGİSİ

Cukurova Med J 2017;42(1):48-54
DOI: 10.17826/cutf.280083

ARAŞTIRMA/RESEARCH

Effect of lead nanoparticles inhalation on mesostructure and the osteoprotegerin/receptor activator of nuclear factor-kappaB ligand system in rats

Sıçanlarda kurşun nanoparçacıkların inhalasyonunun mezoyapı ve nükleer faktör-kappaB ligand sisteminin aktivatör reseptörü olan osteoprotegerine etkisi

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Cukurova Medical Journal 2017;42(1):48-54.

Abstract

Purpose: This study aims to investigate whether Pb nanoparticle exposure affects the mesostructure, and osteoprotegerin/receptor activator of nuclear factor-kappaB ligand (OPG/RANKL) system in rats exposed to subchronic and chronic inhalation.

Material and Methods: Forty eight 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/m3 an hour daily for 28 days. Another three groups were exposed to nanoparticles Pb at following doses 6.25; 12.5; and 25 mg/m3 one hour daily for 6 months.

Results: Subchronic and chronic Pb nanoparticles changed trabecular mesostructure. We found that subchronic exposure significantly increased the levels of OPG at the first and second dose exposure compared to the control groups ($P < 0.05$). In the chronic exposure, we found that the levels of OPG significantly increased at the first and second dose exposure compared to the control group ($P < 0.05$). In this study significant reduction in subchronic exposure to second and third doses compared to the first dose or exposure control ($P < 0.05$) were shown. In chronic exposure, no significant differences in RANKL levels were found between groups ($P > 0.05$). In subchronic exposure, the ratio of OPG/RANKL significantly increased between the third dose exposure compared to controls ($P < 0.05$), with not significant

Öz

Amaç: Bu çalışma, subkronik ve kronik inhalasyona maruz bırakılan sıçanlarda Pb nanopartikül maruziyetinin nükleer faktör-kappaB ligand (OPG / RANKL) sisteminin mezoyapı ve osteoprotegerin / reseptör aktivatörünü etkileyip etkilemediğini araştırmayı amaçlamaktadır.

Gereç ve Yöntem: Kırk sekiz sıçan tesadüfen sekiz gruba ayrıldı. Bir grup, açıklanmamış bir gruptur. Üç grup Pb nanopartiküllere maruz bırakılmışken 6.25; 12.5; veya 28 gün boyunca günde bir saat 25 mg / m^3 uygulandı. Diğer üç grup, nanopartikül Pb'ye maruz bırakıldı; dozlar 6.25 12.5; ve 6 ay süreyle günde bir saat 25 mg / m^3 tü.

Bulgular: Subkronik ve kronik Pb nanopartikülleri trabeküler mezoyapıyı değiştirdi. Subkronik maruziyetin birinci ve ikinci dozu kontrol gruplarına kıyasla OPG düzeylerini önemli ölçüde arttırdığını bulduk ($P <0.05$). Kronik maruziyetinde, OPG düzeylerinin birinci ve ikinci dozu kontrol grubuna göre anlamlı olarak arttığını bulduk ($P <0.05$). Bu çalışmada, subkronik maruziyetinde ikinci ve üçüncü dozları, birinci doza veya kontrol grubuyla kıyaslandığında önemli bir ($P <0.05$) azalma gösterdi. Kronik maruziyette, gruplar arasında RANKL düzeylerinde anlamlı bir farklılık bulunmadı ($P> 0.05$). Subkronik maruziyette, OPG / RANKL oranı üçüncü dozla kontroller kıyaslandığında anlamlı olarak arttı ($P <0.05$) ve kronik maruziyette anlamlı farklılıklar görülmedi.

Sonuç: Osteoblastın RANKL inhibisyonu ve OPG stimülasyonunun enazından bir bölümünün aracılığıyla Pb

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Geliş tarihi/Received: 17.05.2016 Kabul tarihi/Accepted: 19.06.2016

differences in chronic exposure.
Conclusion: Pb nanoparticle induced trabecular bone neoformation at least a part via OPG stimulation and inhibition of RANKL by osteoblast.
Key words: Inhalation, toxicology, bone remodeling, femur.

nanopartikül trabeküler kemik neoformasyonuna indüklendi.

Anahtar kelimeler: İnhalasyon, toksikoloji, kemiğin yeniden oluşumu, femur.

INTRODUCTION

Lead (Pb) is a heavy metal that is more widespread than any other metal. Levels of Pb in the environment increased, as mining, smelting, and a variety of uses in industry. 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. Firstly, Pb can be found in the blood. As much as 95% Pb binds to erythrocytes with a half-life of 25-30 days. Secondly, Pb can be found in soft tissue, with a half-life of several months. Thirdly, Pb can be found in the bone with a half-life of 30-40 years. As much as 90% of Pb that enters the body will accumulate in the bone. Pb follow the path of calcium to enter the cells of the body[2].

Ingestion of Pb orally by mice shows that there is an accumulation of Pb in the tibia compared with those not given the Pb. Exposure to Pb can cause a decrease in somatic growth, longitudinal bone growth and bone strength at the time of puberty. In the end, Pb exposure can inhibit osteoblastogenesis in adult animals[3]. 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 compete 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[4].

Bone remodeling occurs throughout life via synthesis of bone matrix through the action of two major cell types: osteoblasts and osteoclasts[5]. Osteoblasts are responsible for bone formation, while osteoclasts are in charge of bone resorption[6,7]. The proper functioning of these two cell types is necessary for the maintenance of bone mass as well as bone mineral density. Osteoporosis is defined as a reduction in bone mass and the disruption of bone micro-architecture, which results in a decrease in bone strength and an increase in fracture risk[8]. This study aims to investigate whether Pb nanoparticle exposures affects the mesostructure, and osteoprotegerin/receptor activator of nuclear factor-kappaB ligand (OPG/RANKL) system in rats exposed to subchronic and chronic inhalation.

MATERIAL AND METHODS

Animals

Male Wistar albino rats, 16 weeks of age, weighing 175–200 grams, were used for this study. Forty eight 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/m3 an hour daily for 28 days. Another three groups were exposed to Pb nanoparticles at doses of 6.25; 12.5; and 25 mg/m3 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, Indonesia9-12 and Turkey13. 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 PM10 coal dust, which can be inhaled by rats. Chamber size was 0.5 m3 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-exposure group was exposed to filtered air in the laboratory.

Tissue and serum sampling

At the end of the treatment, the animals were euthanized by anesthetizing with ketamin injection. The femur was collected, weighed, and washed with physiological saline. The right femur was histologically processed with a scanning electron microscope. The blood was collected from the cardiac. All femur and serum samples were labeled and stored at −80°C until analysis.

Femur mesostructure

The femoral head was cut with a scalpel. For scanning electron microscopy (SEM) evaluation, femurs from all groups were cut vertically at the femoral head. Then the femoral bones were fixed in phosphate-buffered formalin, dehydrated in graded concentration of ethanol and coated with gold and palladium. The processed bones were then analyzed at 20 kV accelerating voltages by an SEM (FEI Inspect TM S50)12.

Analysis of OPG and RANKL

The serum OPG and RANKL ELISA kit were purchased from USCNK, Life Science, Inc (Wuhan, Hubei, PRC). The analysis was done according to specify procedures in the kit.

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.

RESULTS

Figure 1 presents the mesostructure of each experimental group. The SEM of femur in control (untreated) group subchronic exposure showed a step-ladder pattern of collagen with granule formation covered by the fibrillar collagen (A). In the rats exposed to first dose of subchronic Pb nanoparticles, there were irregular step-ladder patterns of collagen with smooth surface topography, similar thickness of trabeculae with the control group, and minimal granule formation (B). These granules will disappear in the second dose so that the surface becomes flat (C). For the third dose, starting discovered granules and irregular surfaces (D). In the control chronic group was found trabecular thick, flat surface, and still visible collagen fibers (E). In the rats exposed to chronic Pb nanoparticles, there were increasing a trabecular thickness accompanied by a regular step-ladder pattern of collagen with smooth surface topography (F-H).

Figure 2 presents the OPG serum levels in subchronic and chronic exposure groups. The levels of OPG were significantly increased at the first and second dose subchronic exposure compared to the control group ($P < 0.05$). There was no significantly different between the first and second dose of subchronic exposure ($P > 0.05$).

OPG levels significantly decreased in the highest dose exposure compared to the two lowest dose groups ($P < 0.05$), to reach the similar level with the control group ($P > 0.05$). We found significantly increased levels of OPG in the first and second dose chronic exposure groups compared to control group ($P < 0.05$). The levels of OPG showed significantly lower reduction in the highest dose group compared to the second dose group ($P < 0.05$), reaching the levels comparable to the control group ($P > 0.05$).

Figure 3 presents the level of RANKL serum in each experimental group. The RANKL levels significantly decreased in second and third doses subchronic exposure groups compared to the first dose or the control groups ($P < 0.05$).

The levels of RANKL was not significant differences between chronic exposure group ($P > 0.05$).The ratio of OPG/RANKL significantly increased in the second dose of subchronic group compared to the control groups ($P < 0.05$), but not significantly different in chronic exposure, as seen in Figure 4.

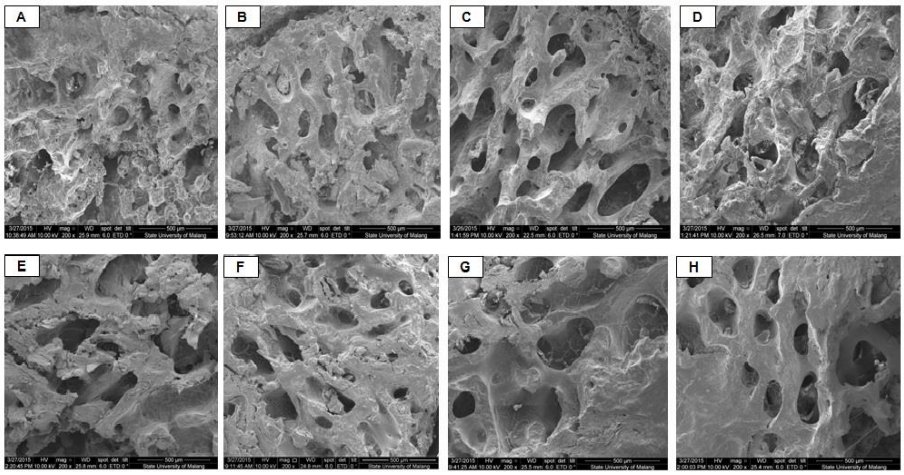

Figure 1. Micrograph illustrating the mesostructure of femur in rats. The SEM of femur in control (untreated) group of subchronic exposure (A) showed a step-ladder pattern of collagen with granule formation covered the fibrilar collagen. The SEM of femur in control (untreated) group of chronic exposure (E) showed a increasing a trabecular thickness without granule formation but covered the fibrilar collagen. In the rats exposed to subchronic Pb nanoparticles (B-D), there was irregular step-ladder pattern of collagen with smooth surface topography. In the rats exposed to chronic Pb nanoparticles (F-H), there was increase in the trabecular thickness with regular a step-ladder pattern of collagen with smooth surface topography. (Scanning Electron Microscope; 10.0 KV; Magnification x2000).

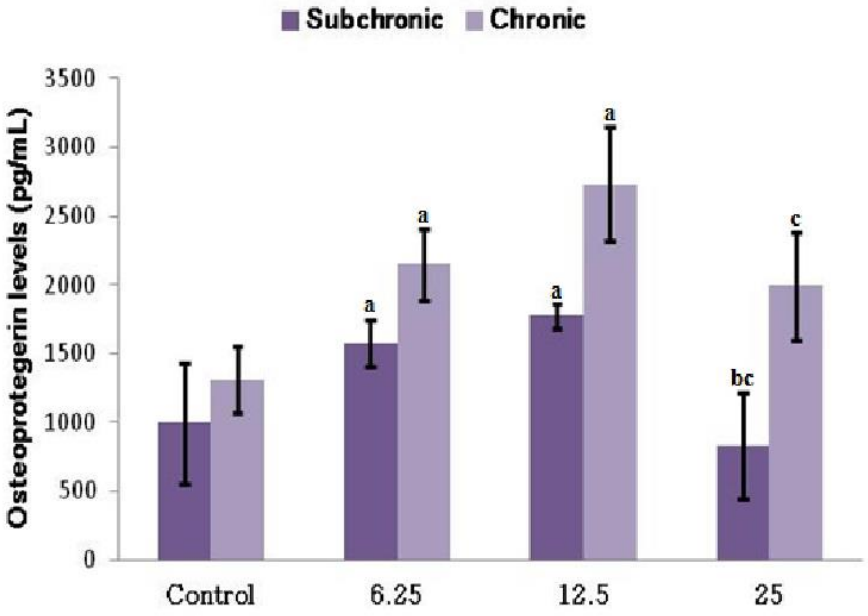

Figure 2. The level of OPG in each experimental groups.

Note: Values are presented as mean ± standard deviations; a $p < 0.05$ compared to the control group; b $p < 0.05$ compared to the group exposed to a dose of 6.25 mg/m3; c $p < 0.05$ compared to the group exposed to a dose of 12.5 mg/m3; pg/mL: picogram/milliliter; blue bar: subchronic exposure; red bar: chronic exposure. Data 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.

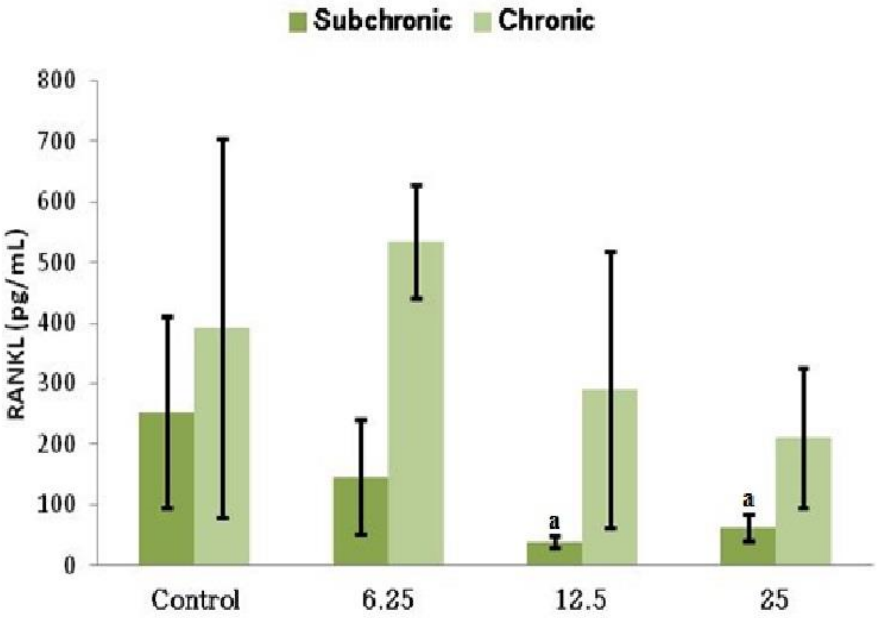

Figure 3. The level of RANKL in each experimental groups.

Note: Values are presented as mean ± standard deviations; a $p < 0.05$ compared to the control group; pg/mL: picogram/milliliter; blue bar: subchronic exposure; red bar: chronic exposure. Data 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.

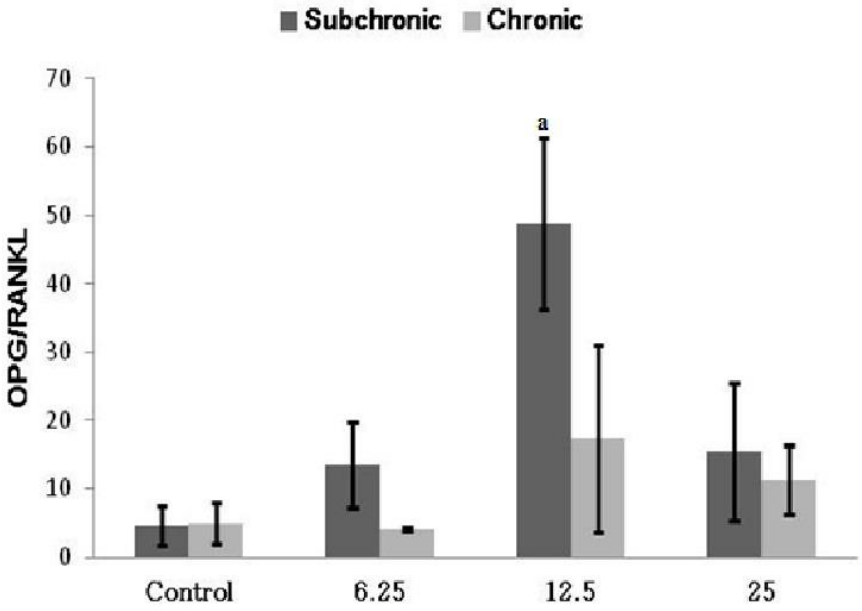

Figure 4. The ratio of OPG/RANKL in each experimental groups.

Note: Values are presented as mean ± standard deviations; a $p < 0.05$ compared to the control group. blue bar: subchronic exposure; red bar: chronic exposure. Data 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.

DISCUSSION

A number of properties, such as thicker wall, well-connected trabeculae, and plate-like trabeculae, confer a better and stronger trabecular bone properties[12, 14-16]. The trabecular region in the femur or tibia may be the optimal region for detecting such bone loss[17]. The differences were found between adults and juveniles respect to this bone compositional variable may suggest that Pb exposure effect in a different way, according to the stage of bone development[18]. The difference in mesostructure of femur in control (untreated) group was found compared with those exposed groups. This regular step-ladder pattern of collagen with granule formation covered the fibrillar collagen in control become irregular step-ladder pattern with smooth surface topography (first and second doses). This surface became granular in high doses. In the rats exposed to chronic Pb nanoparticles, there was increase in the trabecular thickness with a regular step-ladder pattern of collagen with smooth surface topography. Our finding showed that chronic Pb nanoparticles induced trabecular bone growth. Previous studies reported that Pb exposure decreased bone remodelling rate in adult red deer from the mining area. This could be due to reduced activity of bone forming osteoblasts, resulting in a higher relative amount of mature bone mineral (with lower carbon content) compared to newly formed bone mineral (with higher carbon content)[19].

Some experimental studies have also suggested that Pb may affect osteoblast and osteoclast function[20-23]. In this study, the levels of OPG were significantly greater in the two lowest doses subchronic and chronic exposure compared to the control group ($P < 0.05$). This increase would be decreased at the highest dose, reaching the levels comparable to the control group ($P > 0.05$). This finding indicated that Pb nanoparticles may have induced OPG secretion from osteoblast at critical match dose. This lag phase indicates the complexity of biological system[24-25].

RANKL belongs to the superfamily of tumor necrosis factor. It is a significant factor in the osteoclastic development[26]. Its binding to RANK results in the generation of RANKL and osteoclastic differentiation gene expression, which extends osteoclastic activity and increases bone resorption[5].

For subchronic exposure, we found that RANKL levels significantly reduced in the second and third doses compared to the first dose or control groups ($P < 0.05$). Our finding indicated that subchronic Pb nanoparticles exposure inhibit osteoclastic development and subsequent bone resorption.

It was indicated that sites where expression of RANKL predominates (RANKL>OPG) are putative progressive bone resorption, whereas sites where expression of RANKL and OPG is similar (RANKL=OPG), or where OPG is prevalent (RANKL<OPG), are potentially bone neoformation[27,28]. The ratio of OPG/RANKL significantly increased between the third dose exposure compared to controls of subchronic exposure ($P < 0.05$). Our finding indicated that this subchronic dose exposure, Pb nanoparticles induced potentially bone neoformation. In chronic treatment, RANKL levels and the ratio of OPG/RANKL was not found significant differences between treatment groups ($P> 0.05$). During this chronic treatment, we further hypothesized that Pb nanoparticles are involved in homeostatic mechanisms in the OPG/RANKL system by normalizing RANKL level. Previous studies showed that Pb is able to repair the defect of mandibular bone[29].

Overall, the limitations of this study was the absence of a quantitative analysis of the bone mesostructure. It will be of interest in future studies.

In conclusion, Pb nanoparticle induced bone neoformation at least a part via OPG stimulation and inhibition of RANKL by osteoblast. Trabecular bone formation was achieved on subchronic exposure to high doses and all doses of chronic exposure.

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