

Original Article

Effects of subchronic exposure to lead acetate and cadmium chloride on rat's bone: Ca and Pi contents, bone density, and histopathological evaluation

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Abstract: This study aims to investigate the effects of low-dose subchronic exposure to lead acetate ($\text{Pb}(\text{NO}_3)_2$) and cadmium chloride ($\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$) on bone in rats. The rats were assigned randomly to a control group and three experimental groups that were given the mixture of $\text{Pb}(\text{NO}_3)_2$ and $\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$ by gastric gavage at doses of 0 mg/kg body weight (b.w.) (Group I, to serve as a control), 29.96 mg/kg b.w. (Group II, 29.25+0.71), 89.88 mg/kg b.w. (Group III, 87.74+2.14), and 269.65 mg/kg b.w. (Group IV, 263.23+6.42) for at least 90 consecutive days. Calcium (Ca) and phosphorus (Pi) contents in the bone were determined. Bone mineral density (BMD) was measured at the tibia and femur region by dual-energy X-ray absorptiometry. The histopathology of bone was evaluated by light microscope, scanning electron microscope, and transmission electron microscope. The BMD of rats in the experimental group was significantly lower and the contents of Ca and Pi were decreased than those in the control group. The histopathological evaluation showed that co-induction of Pb and Cd results in bone microstructure damage, especially to trabecular bone, marrow cavity, collagen fiber, and osteoblast. In general, results indicate that combining Pb with Cd induces bone damage and increases the risk of osteoporosis.

Keywords: Lead and cadmium, bone mineral density, calcium and phosphorus, bone microstructure, subchronic, rats

Introduction

The environment has been filled with pollutants over the years. People are frequently exposed to two or more kinds of contaminants. These pollutants are recognized for being great harm to public health worldwide; they are natural or anthropogenic sources of pollution to the environment [1]. Lead (Pb) and cadmium (Cd) are widely distributed in the environment because of natural and human-related activities [2, 3]. Exposure to Pb and Cd can lead to damage to several organs, including liver, kidney, and bone [4-6]. Therefore, investigating the effects of the co-administration of Pb and Cd is necessary.

Most animal studies about Pb and Cd toxicity are limited to individual exposure to the harm of bone. Pb mainly accumulates in bones by replacing calcium, and the bones contain approximately 90% of the total amount of Pb in the body [7, 8]. The long-term effects of Pb poi-

soning can lead to reduced bone mineralization [4]. Moreover, exposure to Pb can result in a decrease in bone mineral density (BMD) [9]. The bone is the toxicity target organ of Cd [10]. Moreover, Cd exposure can result in a decrease in trabecular number and thickness [11].

People are frequently exposed to combinations of contaminants [12]. However, the effects of mixed contaminants toxicity lack comprehensive data. Thus, further experiments on the combinations of toxic metals are needed. Most studies about Pb and Cd co-exposure are mainly about organs, such as liver and kidney [5, 12]. However, the data regarding the effects of co-exposure to low doses of Pb and Cd on the bone are little. Therefore, this study exposes animals to Pb and Cd to investigate heavy metal toxicity in bones. Moreover, numerous models have been explored to predict the potential effects of chemical mixture for an individual species or population group.

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In our previous research, lead acetate (Pb (NO₃)₂) and cadmium chloride (CdCl₂·2.5H₂O) were used in an equitoxic mixture ratio design. The fixed mixture ratio was based on the single toxicant LD₅₀-value (median lethal dose value) for an oral acute study [13]. Moreover, the result shows that the acute toxicity of Pb and Cd can lead to bone damage. In this study, people may be exposed to Pb and Cd from industrial sources because of the presence of these elements in the natural environment. Thus, we chose low-dose exposure via the subchronic experiment. The experimental model of rats treated with relatively low Pb and Cd levels are based on the mixture toxicant LD₅₀-value of 2696.54 mg/kg our oral acute study for a 90-day period as a model of Pb and Cd-induced subchronic toxicological evaluation.

The purpose of this study is to evaluate the effect of subchronic Pb and Cd co-exposure on bone and its mechanism by examining the content of Ca and Pi in bone and BMD, and through histopathological evaluation.

Materials and methods

Chemicals and animals

Analytical grade Pb (NO₃)₂ and CdCl₂·2.5H₂O (AR>99.0%) were obtained from Sigma Aldrich (St. Louis, MO, USA). The study was carried out with Sprague-Dawley rats with an initial body weight of approximately 150 g. All rats were obtained from Chengdu Dossy Experimental Animals Co., Ltd. (License No. SCXK (Sichuan) 2008-24, China) and kept in the animal house at the Sichuan Agriculture University (Ya'an, China). The animals were housed under controlled conventional conditions (temperature 25±3°C, relative humidity of 35% to 60%, 12-hour light-dark cycle) and allowed free access to a standard rat chow and drinking water during the experiment. The experimental protocol was approved by the Local Ethics Committee for Animal Experiments in Sichuan Agricultural University (China) for the care and use of laboratory animals.

Experimental protocol

All animals took 7 days to adapt to the experimental conditions prior to the experiment. The rats were divided randomly into four groups, that is, one control group and three experimen-

tal groups; each group has 20 rats. Experimental groups were treated with a mixture of Pb (NO₃)₂ and CdCl₂·2.5H₂O solution at dose of 29.96 mg/kg body weight (b.w.) (Group II, 29.25+0.71), 89.88 mg/kg b.w. (Group III, 87.74+2.14), and 269.65 mg/kg b.w. (Group IV, 263.23+6.42) by gastric gavage for at least 90 consecutive days. The control group was treated with a solution free of Cd and Pb. In each case, the volume of the reagent, which was administered to the rats by gavage is 10 mL/kg b.w. Each rat was marked with a unique identification number by trinitrophenol.

After the exposure period (90 days) of the experiment, six rats were randomly selected from each group. All rats were anesthetized with diethyl ether. Rats that fasted were sacrificed by femur and tibia dissection under ether anesthesia. Left femur and tibia were isolated from soft tissues for the measurement of BMD, as well as Ca, Pi, Pb, and Cd content. The right femur was obtained for bone histopathology.

Analysis of Ca, Pi, Pb, and Cd contents in bone

The bone sample was dried to constant weight (dry weight) at 80°C for 48 hours. The dried sample was ground into powder and placed in 10-mL conical flasks containing 5 ml of nitric acid overnight. The sample was digested with 2 ml of 30% H₂O₂ with a microwave system every other day. Ca and phosphate (Pi) contents in the preparations of bone were measured by inductively coupled plasma mass spectrometry (ICP-MS; SPECTRO ARCOS; Spectro Analytic Instruments GmbH, Germany). The bone sample was then analyzed for Pb and Cd content with atomic absorption spectrometry (AAS, SPECTRAA 220FS; Varian Associates Inc., USA).

Bone densitometry

BMD was measured at the tibia and left femur region by dual-energy X-ray absorptiometry (DEXA) with a Lunar® DPX-L (USA) densitometer with small animal software. All samples measured three times the average record. All densitometrical parameters were analyzed by the same examiner on the same day.

Bone histopathological evaluation

Optical microscopy observation: Hematoxylin and eosin (HE) stain was used in the histology

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Table 1. Effects of subchronic co-exposure to lead acetate and cadmium chloride on Ca and Pi contents in bone of rats

Groups	Doses (mg/kg/day)	Ca (mg/g)	Pi (mg/g)
Group I	0	103.50±3.41	67.50±2.95
Group II	29.96	96.83±2.60	63.33±1.26
Group III	89.88	93.67±2.30*	60.17±2.24*
Group IV	269.65	89.17±1.33**	59.00±2.11*

Group I: Control group; Group II: Low dose group; Group III: Intermediate dose group; Group IV: High dose group. Data are shown as means ± SE (n=6). Statistically significant differences are indicated by *p<0.05, and **p<0.01, vs. control.

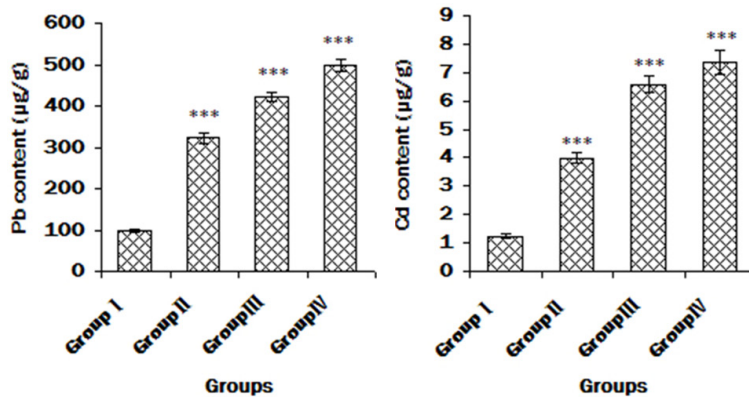


Figure 1. The content of Pb and Cd in the bone. Group I: Control group; Group II: Low dose group; Group III: Intermediate dose group; Group IV: High dose group. Data are shown as means ± SE (n=6). Three asterisks indicates statistically significant difference (P<0.001) vs. control.

Table 2. Effects of subchronic co-exposure to lead acetate and cadmium chloride on bone density (BMD) of rats

Groups	Doses (mg/kg/day)	Femur (g/cm ²)	Tibia (g/cm ²)
Group I	0	0.183±0.004	0.150±0.003
Group II	29.96	0.174±0.005	0.137±0.003
Group III	89.88	0.155±0.006**	0.134±0.007*
Group IV	269.65	0.147±0.008**	0.129±0.004**

Group I: Control group; Group II: Low dose group; Group III: Intermediate dose group; Group IV: High dose group. Data are shown as means ± SE (n=6). Statistically significant differences are indicated by *p<0.05, and **p<0.01, vs. control.

analysis. The right upper femur was fixed in 4% paraformaldehyde for three days at 4°C and decalcified in 10% ethylenediaminetetraacetic (EDTA) acid at room temperature for three days. The femur was dehydrated through a series of ascending ethanol solutions (40% to 100%), cleared with dimethylbenzene, enclosed in paraffin, and sliced with 5-mm thickness with rotary microtome (Leica RM 2255, Leica Instruments Ltd., Germany) for HE stain. Finally, photos were taken with a microscope for analysis (Nikon 80i, Nikon Corporation, Japan).

Scanning electron microscopy observation: The femur fragments were cut in small squares with a saw and kept in 2.5% glutaraldehyde after washing in a buffer solution for a few minutes to remove any debris. The bone was mounted on the scanning electron microscope (SEM) stubs with carbon tape and was carbon-coated. The sample was studied and photographed with a Hitachi S520 SEM (Tokyo, Japan).

Transmission electron microscopy observation: The cancellous bone from femur metaphysis of rats was fixed in 2.5% glutaraldehyde at 4°C for 24 hours and decalcified in EDTA acid for three weeks at room temperature. The bone was cleared with PBS, fixed in 1% osmium tetroxide, dehydrated through a series of ascending ethanol solutions, embedded in epoxy resin 618, and sliced for uranyl acetate and chromic acid Pb double staining. The compact bone of the same size from femoral shaft was fixed in 2.5% glutaraldehyde for 24 hours at 4°C, cleared with PBS, soaked in saturated sucrose solution overnight, and cut into frozen ultrathin section by microtome (Leica EM6/UC6, Germany). Ultimately, the changes in osteocyte ultrastructure, collagenous fibers,

and bone matrix were observed under transmission electron microscope (TEM) (JEM-1010, Joel, Japan).

Statistical analysis

All statistical analyses were performed with SPSS 17.0 software. Data were expressed as mean ± SE (x ± s) for the number of experiment. The significance level of P<0.05(*), P<0.01(**) or P<0.001(***) were represented as asterisks. The statistical analyses were carried out with ANOVA, and the analysis of group differ-

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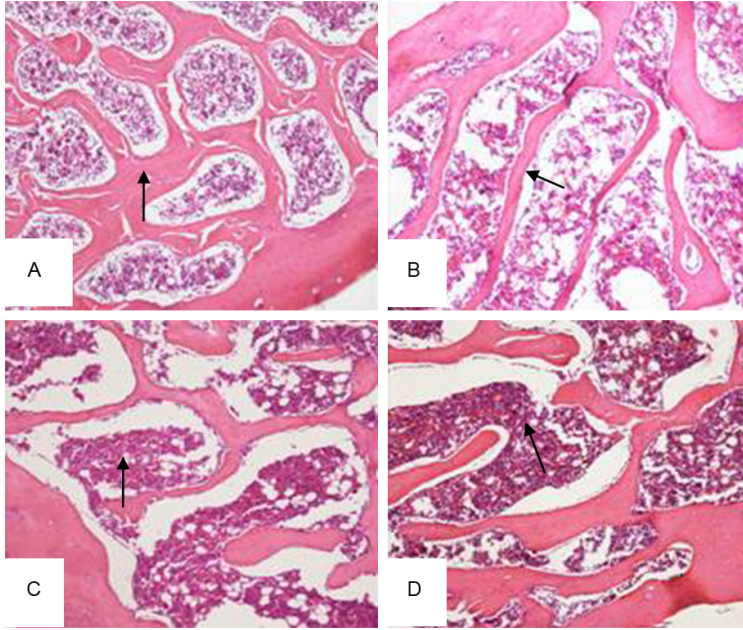


Figure 2. Effects of subchronic co-exposure to lead acetate and cadmium chloride on the structure of rats bone under the optical microscope for 90 d. A: The control group shows the normal structure of trabecular (↑) (HE×100); B-D: The trabecular bone was less, thinner (↘) and fractured (↑), marrow cavity (↑) are expanded into irregular shapes in the experimental group (HE×100).

ences was performed by the Student-Newman-Keul's test.

Result

Ca and Pi content in bone

At the 90th day, Ca and Pi content in bone were determined by ICP-MS (**Table 1**). The Ca and Pi contents in the bone of vehicle-control group (Groups I) were higher than those in other groups. Pb and Cd co-exposure for Groups III and Group IV were decreased ($P<0.05$ and $P<0.01$, respectively) by Ca. Pb and Cd co-exposure administration (Groups III to IV) caused a significant ($P<0.01$) decrease in Pi.

Femur accumulation of the Pb and Cd

Pb and Cd contents were determined by AAS, and the results are shown in **Figure 1**. In control group (Groups I), the Pb and Cd content in bone is relatively low. Co-exposure to Pb and Cd caused an obvious increase in the accumulation of these elements. Pb and Cd contents in the bone gradually increased with increased doses of ingested Pb and Cd. The content of Pb and Cd in bone is much higher in exposure

group (Groups II to IV) than in control group ($p<0.001$).

BMD of the femur and tibia

The BMD of the femur and tibia was determined by DEXA (**Table 2**). Compared with the control group, the BMD of rats that have been exposed to Pb and Cd (Group III and Group IV) was significantly decreased ($P<0.01$) in the femur. The BMD in the tibia in Group III and Group IV ($P<0.05$ and $P<0.01$, respectively) was decreased in the 90-day period.

Bone histopathological findings

Under optical microscopy, the femoral of the control group rats has many connected trabecular bones. Marrow cavities are separated and have a certain shape (**Figure 2A**). The bone tissue of test rats shows less and thinner trabecular bones than the rats in the control group; the trabecular bones are also fractured. Moreover, the marrow cavity of test rats expanded into irregular shapes. The density of the cortical bone of test rats is less than that of the ones in the control group (**Figure 2B-D**).

Under SEM, the bone trabeculars from the distal of femur in the control group were numerous and displayed uniformity and solid reticulation (**Figure 3A**). However, the bone trabeculars became few and thin as their spacing increases because of exposure to Cd and Pb for 90 days, and their lacunae were in every size and every shape, coupled with destroyed solid reticulation (**Figure 3B**). At high magnification, the surface of bone trabeculars of rats in the control group is smooth, and the collagen fibers disposed tightly and orderly (**Figure 3C, 3D**). However, the surface of bone trabecular of rats from the experimental group is less smooth than that in the control group, and the bone lacunae, which mostly were orbicular or elliptic-spatulate and shallower, were formed (**Figure 3E**). Moreover, the collagen fiber of bone trabecular is in chaos and loose structure and prone to breakage (**Figure 3F**).

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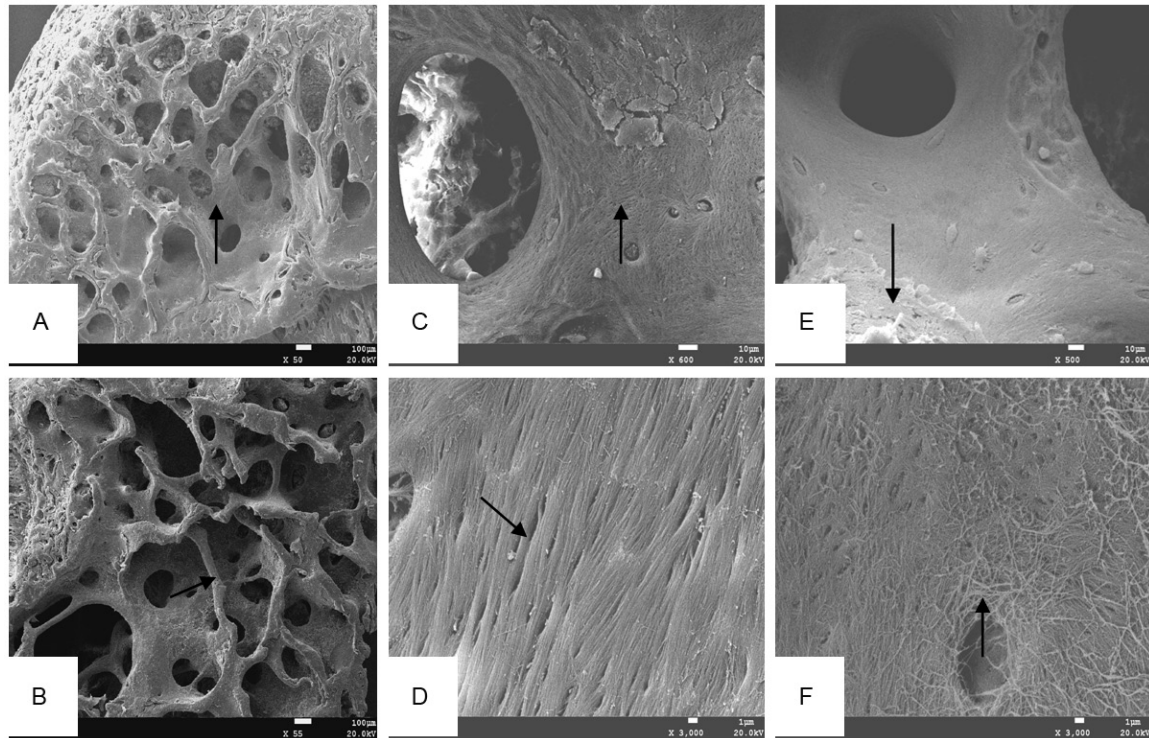


Figure 3. Effects of subchronic co-exposure to lead acetate and cadmium chloride on the structure of rats bone under the scanning electron microscopy for 90 d. A: The control group shows the bone trabeculas from distal of femur were more numerous (\uparrow) ($\times 50$); B: The femoral bone trabecula were few, thin, and developed local fracture (\nearrow) ($\times 55$); C: The surfaces of bone trabeculas from control group were smooth (\uparrow) ($\times 600$); D: The collagen fibers disposed tight and orderly (\searrow) ($\times 3000$); E: The femoral trabecular partly showed erosive bone destruction and there were lacunae on the surface of collagen fibers (\searrow) ($\times 500$); F: The collagen fiber of trabecular fiber arranged in disorder, loose structure and breakage (\searrow) ($\times 3000$).

Under TEM, the control group has rich organelles and evenly distributed chromatin (**Figure 4A**). The ultrastructure changes showed fewer organelles from osteoblast, focal cytolysis, condensation, and margination of chromatin, coupled with greater bone lacunae and more floc in experimental group (**Figure 4B**). The collagen fiber from the control group appeared in an orderly arrangement, and hydroxyapatite crystal, which is tiny acicular, regularly clings to the fiber (**Figure 4C, 4D**). By contrast, the collagen fiber is in chaos and loose structure and has shown signs of breakage and dissolution, coupled with disappeared transversus striation, after the decalcification (**Figure 4E**). Moreover, the experimental group, without decalcification, showed decreased amount of hydroxyapatite crystal and disorder (**Figure 4F**).

Discussion

This study is the first attempt to report the equitoxic mixture ratio of Pb and Cd as the experi-

mental animal model method for subchronic toxicity experiments. Moreover, this animal model may help detect the early events of chronic Pb and Cd intoxication with relatively low and environmentally realistic concentrations. The results indicated that the co-exposure to Pb and Cd, which accumulate in the body, can induce bone damage via change in Ca and Pi content in bone and decrease in BMD and bone morphology lesions.

The bone depression may be due to the absorption of Ca, which is reduced by Pb exposure in rats [14]. Moreover, Cd exposure can inhibit the gastrointestinal absorption of Ca and Pi by competing with its uptake [15]. In addition, Pb mainly replaces calcium hydroxyapatite to form Pb phosphate deposition combined with the hydroxyapatite bone [16]. Exposure to Cd can inhibit hydroxyapatite formation and the Cd^{2+} ions competition with Ca^{2+} ions for incorporation into bone [17]. We observed that co-exposure to low levels of Pb and Cd for 90 days

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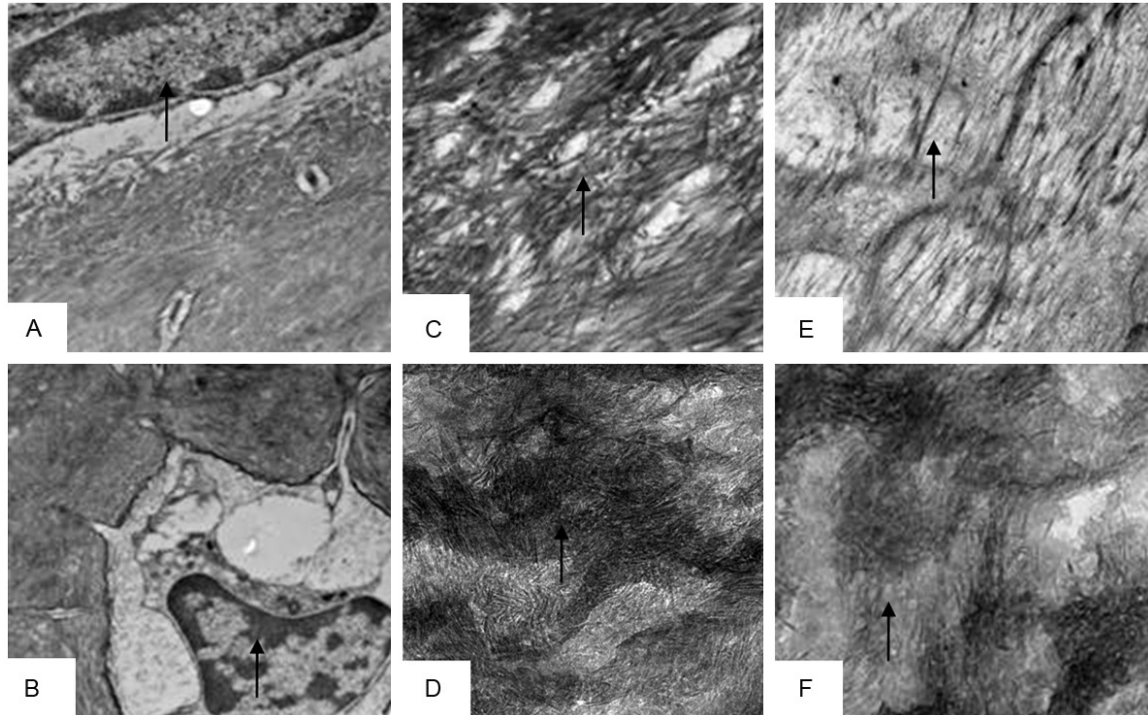


Figure 4. Effects of subchronic co-exposure to lead acetate and cadmium chloride on the structure of rats bone under the transmission electron microscopy for 90 d. A: The control group had rich organelles and evenly distributed chromatin (↑) ($\times 2500$); B: The osteoblast showed fewer organelles, focal cytolysis, condensation and margination of chromatin, coupled with greater bone lacunae and more floc (↑) ($\times 2500$); C, D: Collagen fiber from control group appeared in an orderly arrangement (↑) ($\times 5000$), and hydroxyapatite crystal, which appeared to be tiny acicular, regularly cling to the fiber (↑) ($\times 65000$); E: Collagen fiber arranged in disorder, loose structure, breakage, and dissolution were noted (↑) ($\times 5000$); F: The amount of hydroxyapatite crystal was decreased and arranged in disorder (↑) ($\times 65000$).

result in a significant decrease in Ca and Pi content, as indicated by a marked increase in Pb and Cd in bone with the increasing doses of ingested Pb and Cd. Therefore, these results indicate that Pb and Cd exposure can impair calcium and phosphorus absorption, displace calcium with these metals, and decrease bone mineralization, which interferes with the normal ossification.

BMD is an indicator for diagnosing osteoporosis; this parameter provides information regarding the quantity of mineral in bone, which is only one component of bone strength [18]. Research has shown that calcium deficiency can reduce BMD and weaken bone strength and long-term calcium deficiency can lead to osteoporosis [19]. Moreover, Pb exposure can inhibit the development of bone and reduce BMD because the change in the distribution of elements in the bones [20]. This mineral imbalance can cause osteoporosis because of Cd exposure [21]. Exposure to Cd may be an impor-

tant contributing factor to lower BMD and osteopenia, which result in the development of osteoporosis [12]. In the present study, the BMD was significantly decreased in Pb and Cd-exposed rats. With repeated administration, the content of Ca and Pi deficiency in bone, which indicates co-exposure to Pb and Cd, results in an increased risk of osteoporosis.

Osteoporosis can cause incremental bone fragility and increased fracture risk [22, 23]. Moreover, bone microstructure is an important factor in understanding the mechanisms of bone fragility. In this study, co-exposure to Pb and Cd can induce bone microstructure damage. Pb plays an important role in the development of bone pathologies [24]. Moreover, Pb can cause decreased trabecular bone of rats and irregularity in the primary bone marrow cavity [14]. When Cd accumulates in the body, it can induce damage to the cancellous bone microstructure [25]. Collagen fibers constitute

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an organic matrix where minerals are incorporated while creating the bone tissue. Moreover, collagen fibers are responsible for bone elasticity. In addition, the osteoblast participates in ossification. The results showed that trabecular bone becomes fewer, the collagen fiber of bone trabecular is arranged in a disorderly and loose structure, which is prone to breakage, and the osteoblast showed fewer organelles, focal cytolysis. Therefore, these results indicate that co-exposure to Pb and Cd interferes with the normal ossification and increased fracture risk, thus leading to osteoporosis.

In summary, exposure to Pb and Cd can induce bone damage and leads to an increased risk of osteoporosis. The effects produced by the combined treatment of metals were not only the inhibition of Ca and Pi deposit and the reduction of bone density, but also severe bone morphology lesions.

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Disclosure of conflict of interest

None.

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