

Original Article

Effects of mixed subchronic lead acetate and cadmium chloride on bone metabolism in rats

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Abstract: This study aimed to determine the effects of administering a mixture of subchronic lead acetate (Pb (NO₃)₂) and cadmium chloride (CdCl₂·2.5H₂O) on the bone metabolism of rats. A control group and three experimental groups consisted of randomly selected rats. Rats in each experimental group were orally administered with a mixture of Pb (NO₃)₂ and CdCl₂·2.5H₂O with the following respective doses for 90 consecutive days: 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). Serum osteocalcin (OC) and bone-specific alkaline phosphates (BALP) were considered as bone-formation markers, whereas carboxy-terminal cross-linking telopeptides of type I collagen (CTX) in serum acted as bone resorption markers. Calcitonin (CT) and parathormone (PTH) were tested as calciotropic hormones markers. The (Ca) and phosphorus (Pi) concentrations in the serum and urine were determined. These results were indicated by a significant ($P < 0.05 - P < 0.01$) increase in BALP, CTX, and PTH concentrations and decrease in CT and OC concentrations. Moreover, the concentrations of Ca and Pi in the serum were decreased, whereas those in urine increased. Results indicated that the administration of Pb and Cd induced bone metabolism disorders by decreasing bone formation and increasing bone resorption to destroy the hormonal regulation of mineral metabolism as a result of Ca and Pi imbalance.

Keywords: Lead and cadmium, calcium and phosphorus, bone metabolism, calciotropic hormones, rats

Introduction

Heavy metals are natural or anthropogenic sources of pollution to the environment, and they pose great harm to public health worldwide [1, 2]. Two of the most abundant heavy metals that often coexist in the environment are lead and cadmium [3]. Exposure to these metals can lead to damage of several organs: liver, kidney, bone [4, 5]. Therefore, the effects induced by Pb and Cd must be investigated.

Pb and Cd can be accumulated in the human body through a variety of pathways: respiration, adsorption, and ingestion of cigarette smoke; skin; food [6, 7]. The bone is the target of toxic Pb and Cd [8, 9]. These metals accumulate in the skeleton during one's lifetime and affect the bone tissue by directly influencing the hydroxyapatite formation and the bone cell activity, as well as indirectly by disturbing the mineral metabolism [8, 10]. Pb and Cd can compete with Ca for incorporation into the

bone, and they inhibit the hydroxyapatite formation [11, 12]. The maintenance of bone mass is known to depend on the equilibrium between the bone formation and the bone resorption because of the osteoclastic activity [13]. Pb might have some specific toxicity on osteoblasts, resulting in adverse effect on the markers of osteoblasts [14]. Furthermore, exposure to Cd can disrupt the bone metabolism, which is reflected mainly in the changes in the rate of the bone turnover [15]. Pb is a divalent cation and can displace calcium in the bone matrix because of physical and chemical characteristics similar to those of Ca [16, 17]. Moreover, exposure to Pb can perturb cellular calcium homeostasis, alter sizes and flux rates of cellular pools of exchangeable Ca, and impair Ca-mediated cell processes [18]. In addition, studies have demonstrated that exposure to Cd may disrupt the calcium metabolism, resulting in the increase of urinary calcium excretion [19].

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In most previous animal studies, only one type of metal has been used in high concentration. However, people are actually frequently exposed to combinations of contaminants in the environment [20], and data on the effects of mixed metals are lacking. Thus, further research is needed to investigate the influence of co-contaminants of toxic metals on human exposure at sub-chronic concentrations.

We previously conducted acute toxicity studies that involved lead acetate ($\text{Pb}(\text{NO}_3)_2$) and cadmium chloride ($\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$), which were based on a single toxicant LD_{50} -value for an equitoxic mixture ratio design [21]. The result showed that exposure to Pb and Cd had certain toxic effects on the bone of rats. Because Pb and Cd occur in nature, people may be exposed to them from industrial sources. In this study, we chose a low-dose exposure through sub-chronic experiments. Experimental rats were treated with relatively low levels of Pb and Cd, based on a mixture toxicant LD_{50} -value of 2696.54 mg/kg in our oral acute study for a 90-day period (Pb- and Cd-induced subchronic toxicological evaluation).

The present study aimed to investigate the effect of exposure to Pb and Cd on bone metabolism. For this purpose, markers of bone formation and resorption were determined to estimate the rate of bone turnover, and the mineral status of the serum and urine, including Ca and Pi concentrations, was evaluated. Moreover, to explain the possible mechanisms of Pb and Cd action on bone metabolism, calciotropic hormones as well as Pb and Cd concentrations in the blood and urine were determined.

Materials and methods

Chemicals and animals

Analytical grade (AR > 99.0%) of lead acetate [$\text{Pb}(\text{NO}_3)_2$] and cadmium chloride ($\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$), obtained from Sigma Aldrich, were used. Tests were conducted on 160 male and female Sprague-Dawley rats with an initial body weight of 150 g. The rats were provided by Chengdu Dossy Experimental Animals Co., Ltd (License No. SCXK (Sichuan) 2008-24, China). In the animal house at the Sichuan Agriculture University (Ya'an, China), the rats were kept at controlled conventional conditions (temperature $25 \pm 3^\circ\text{C}$, relative humidity of 35% to 60%, 12 h

light-dark cycle) and given a standard rat chow and drinking water in the course of the experiment. Regulations of Animal Experimentation of the College of Veterinary Medicine, Sichuan Agricultural University, based on the Guidelines of the International Committee on Laboratory Animals, were observed.

Experimental design

Before doing the experiment, the rats were acclimatized to laboratory conditions for 7 days. They were randomly assigned to one control group and three groups exposed to Pb and Cd. The control group (Group I) received no treatment. Each of the three exposed groups was respectively treated with aqueous solutions of $\text{Pb}(\text{NO}_3)_2$ and $\text{CdCl}_2 \cdot 2.5\text{H}_2\text{O}$ by gastric gavage at a 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) for at least 90 consecutive days. The volume of the reagent, which was administered to the rats by gavage, was 10 mL/kg b.w. Trinitrophenol was used to mark each rat with a unique identification number.

After exposing each of the three groups to Pb and Cd for 90 days, six rats of each group were randomly selected. The rats were housed in individual metabolic cages for 24-h urine collection. The urine was centrifuged immediately after collection, and its volume was recorded. Finally, after starving the rats overnight, they were sectioned under anesthesia with anhydrous ether. Whole blood samples from the heart were collected, and some of them placed in ion-free tubes treated with 10% nitric acid for Pb and Cd analyses. The other part of the blood without anticoagulant was collected and immediately centrifuged for 5 minutes at 3000 r/min after coagulation, and the serum was separated. The biological material not used immediately was stored frozen at -70°C until it was assayed.

Assay for markers of bone turnover in serum

Osteocalcin (OC) and bone-specific alkaline phosphates (BALP) were determined as bone-formation markers, whereas carboxy-terminal cross-linking telopeptides of type I collagen (CTX) were measured as bone resorption markers. OC, BALP, and CTX were measured with

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Table 1. Effects of subchronic lead and cadmium co-exposure on bone turnover of rats

Groups	Doses (mg/kg/day)	S-OC (ng/ml)	S-BLAP (U/L)	S-CTX (pg/ml)
Group I	0	5.95 ± 0.29	58.33 ± 8.33	57.50 ± 3.61
Group II	29.96	5.37 ± 0.37	70.83 ± 11.02	53.33 ± 2.08
Group III	89.88	4.60 ± 0.49*	79.17 ± 4.17	51.25 ± 3.61
Group IV	269.65	3.62 ± 0.32**	87.50 ± 7.22*	47.08 ± 2.08*

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.

Table 2. Effects of subchronic lead and cadmium co-exposure on calciotropic hormones in serum of rats

Groups	Doses (mg/kg/day)	CT (pg/ml)	PTH (pg/ml)
Group I	0	112.37 ± 2.31	19.25 ± 1.46
Group II	29.96	107.43 ± 2.26	24.40 ± 1.41
Group III	89.88	103.24 ± 3.93*	25.26 ± 1.79*
Group IV	269.65	102.42 ± 2.02*	27.72 ± 2.45*

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 * $P < 0.05$ vs. control.

enzyme-linked immunosorbent assay (ELISA) kits (Wuhan Xinqidi Biological Technology Co., Ltd), an instrument for the BIO-RAD microplate reader. The intra-assay CV for these measurements was ≤ 12%; the inter-assay CV was ≤ 8%.

Assay for calciotropic hormones in serum

The concentration of CT in the serum was determined by utilizing radioimmunoassay (RIA) kit (Beijing of Chinatown force Biotechnology Research Institute). The intra-assay CV was < 1.2%. The serum-intact PTH was measured by using radioimmunoassay (RIA) kit (Diasorin Inc, USA). The intra-assay CV was < 6%. The instrument used was Wallac1480 WIZARD ® 3 Gamma counter (PerkinElmer Finland) γ -counter.

Determination of Ca and Pi concentrations in serum and urine

The serum and urine samples were wet-digested with trace-pure concentrated nitric (HNO_3 , 69%) and hydrochloric (HCl, 36%) acids (Merck, Darmstadt, Germany) using a microwave system and diluted with ultra-pure water. Ca and Pi

concentrations in such preparations of serum and urine were measured by inductively coupled plasma mass spectrometry (ICP-MS; SPECTRO ARCOS; Spectro Analytic Instruments GmbH, Germany).

Determination of Pb and Cd concentrations in blood and urine

The collected whole blood and urine samples were digested with 10% HNO_3 (1 mL of samples: 2.5 mL of HNO_3 , GR grade, ≥65%, Xilong, Chengdu, China) for 3d in a clean room hood. The supernatants were then used to detect Pb and Cd. These samples were determined by atomic absorption spectrometry (AAS, SPECTRAA 220FS; Varian Associates Inc., USA) with flame atomization in an air-acetylene burner and automatic dosage, according to the techniques reported previously [10]. Stocks of standard Cd and Pb solution assigned for AAS (Sigma, St. Louis, MO, USA) were used.

Statistical analysis

All statistical analysis was performed by using SPSS 17.0 software. Data were expressed as mean ± standard error ($\bar{x} \pm s$) for the number of experiments. The significance level of $P < 0.05$ (*) or $P < 0.01$ (**) were represented as asterisks. The statistical analyses were carried out by using One-way Analysis of Variance (ANOVA).

Results

Effect of co-exposure to Pb and Cd on bone turnover

Markers of bone formation and resorption concentration changes are shown in **Table 1**. Compared with the control group, Groups III and IV co-exposed to Pb and Cd showed a decrease ($P < 0.05$ and $P < 0.01$, respectively) in OC. Groups IV co-exposure to Pb and Cd increased ($P < 0.05$) BLAP and CTX.7.

Effect of co-exposure to Pb and Cd on serum concentrations of calciotropic hormones

Changes in the concentration of CT and PTH as calciotropic hormones markers as a result of exposure to Pb and Cd are shown in **Table 2**.

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Table 3. Effects of subchronic lead and cadmium co-exposure on Ca and Pi concentrations in serum of rats

Groups	Doses (mg/kg/day)	Ca (mg/g)	Pi (mg/g)
Group I	0	25.90 ± 1.15	388.50 ± 13.57
Group II	29.96	22.10 ± 1.21*	344.00 ± 10.39*
Group III	89.88	21.95 ± 1.07*	341.50 ± 11.26*
Group IV	269.65	19.85 ± 1.95**	334.00 ± 12.12*

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.

Table 4. Effects of subchronic lead and cadmium co-exposure on Ca and Pi concentrations in urine of rats

Groups	Doses (mg/kg/day)	Ca (mg/g)	Pi (mg/g)
Group I	0	6.85 ± 0.38	452.17 ± 17.22
Group II	29.96	7.65 ± 0.26	493.33 ± 19.64
Group III	89.88	8.15 ± 0.20*	508.50 ± 11.26*
Group IV	269.65	8.35 ± 0.32**	520.67 ± 14.99*

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.

The concentration of CT was decreased, whereas PTH concentration was increased. After 90 d of exposure to Pb and Cd (Groups III to IV), the CT concentration in the exposed rats decreased ($P < 0.05$) compared with that in the control group; by contrast, the PTH concentration increased ($P < 0.05$).

Effect of co-exposure to Pb and Cd on Ca and Pi concentrations in serum and urine

On the 90th day, Ca and Pi concentrations in the serum and urine were determined by ICP-MS (Tables 3 and 4). Compared with the control group, Groups II and III co-exposed to Pb and Cd showed decreased ($P < 0.05$) concentrations; Group IV showed a significant ($P < 0.01$) decrease in Ca in the serum. The serum Pi concentration was decreased ($P < 0.05$) by Pb combined with Cd (Groups II to IV). Pb and Cd co-exposure administration for Groups III and IV resulted in a decrease ($P < 0.05$ and $P < 0.01$, respectively) in Ca of the urine. The urine Pi concentration was decreased ($P < 0.05$) by Pb combined with Cd (Groups III to IV).

Concentration of Pb and Cd in blood and urine

Pb and Cd concentrations were determined by AAS; the results are shown in Figure 1. In control group (Groups I), Pb and Cd concentrations in the blood and urine are relatively low. Pb and Cd concentrations in the blood and urine gradually increased with increased doses of ingested Pb and Cd. The concentration of Pb and Cd in the blood and urine in the exposed group (Groups II to IV) is much higher than that in the control group ($P < 0.001$).

Discussion

Pb and Cd could affect the bone, but no study has observed the combined effects of Pb and Cd on bone metabolism. This study is the first attempt to report the equitoxic mixture ratio of Pb and Cd based on an experimental animal model method for subchronic toxicity experiments. The results indicated that co-exposure to Pb and Cd, which were distributed in the blood as well as in the urine, can disturb the bone metabolism by affecting the bone turnover process and change calciotropic hormones concentration and disrupt the Ca and Pi balance.

Bone undergoes continuous remodeling consisting of osteoclastic resorption of old bone and osteoblastic synthesis of new bone organic matrix and its mineralization [22, 23]. The balance between bone formation and resorption was disturbed because of exposure to Pb and Cd, resulting in serious consequences for the bone health. In this study, we observed that exposure to a low dose of Pb combined with Cd resulted in increased bone resorption and inhibited its formation, which indicated that the rate of bone turnover enhanced as well as the bone mineral status disturbed.

The mechanisms explain the Pb and Cd effect on the bone metabolism. The decreased activity of osteoblasts inhibited the process of bone synthesis because of the decrease in OC concentrations in the serum after co-exposure to Pb and Cd. Pb^{2+} can displace Ca^{2+} from osteocalcin, and it has a much higher affinity for osteocalcin than that of Ca^{2+} [24]. Moreover, Pb could inhibit the combination of osteocalcin and hydroxylapatite, which leads to the inactivation of osteocalcin [25]. OC had a significantly reduced result from the inhibitory effect

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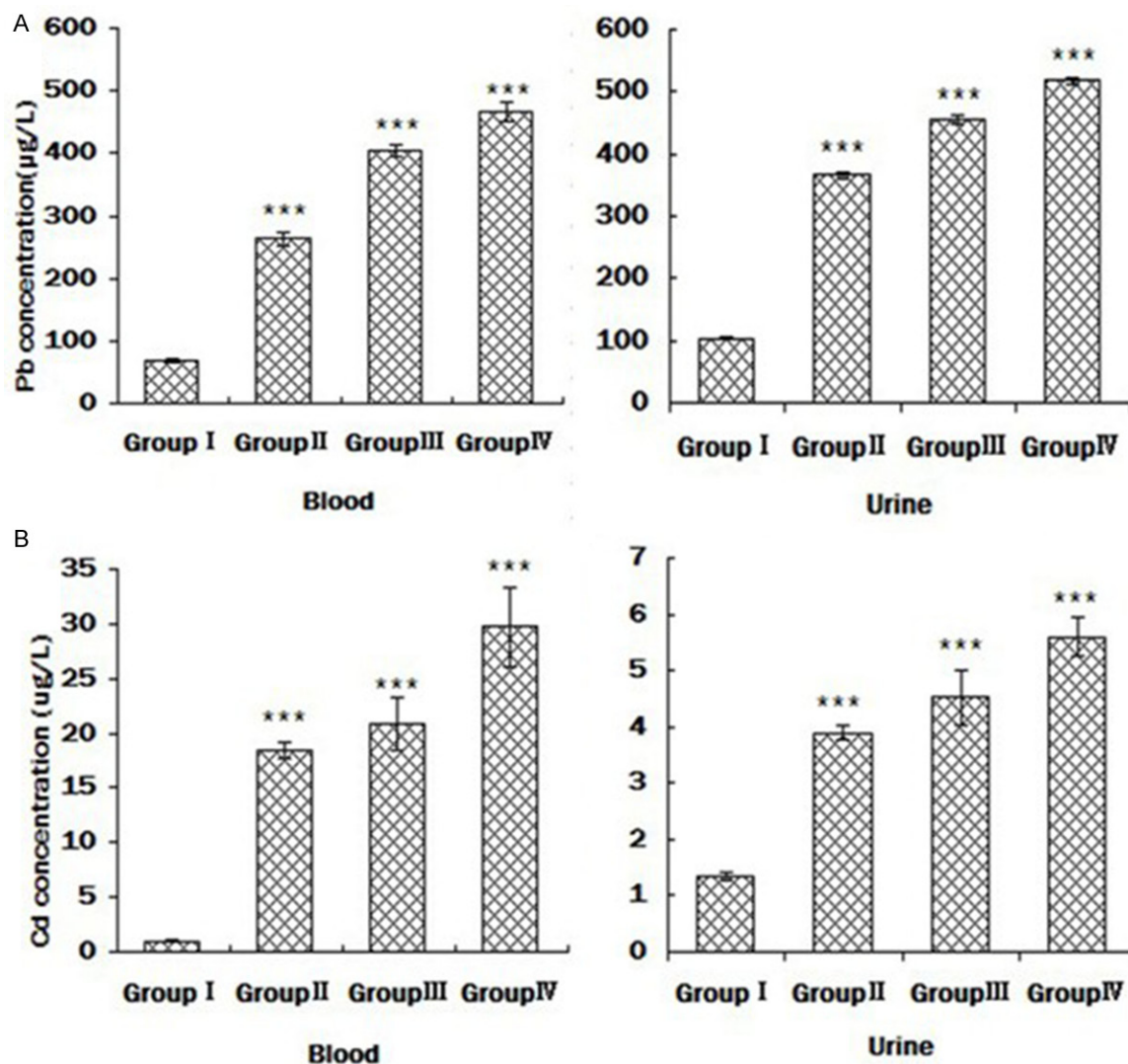


Figure 1. The concentrations of Pb (A) and Cd (B) in the blood and urine. Group I: control group; Group II: low dose group; Group III: intermediate dose group; Group IV: high dose group. Data are shown as means \pm SE (n = 6). Three asterisks indicate statistically significant difference ($P < 0.001$) vs. control.

induced by Cd on the osteoblasts in the bone marrow cells [26].

BALP can effectively monitor the rate of bone mineralization. Different levels of Pb can affect the metabolism of BALP [27]. With increasing blood lead level, the serum OC had the tendency to decline, but BALP had the tendency to increase, which affected the function of osteoblast and inhibited the degrees of bone mineralization [28]. Cd caused the increased concentration of BALP in the serum [29]. In the present study, BALP concentration in Pb- and Cd-exposed rats increased. The results indicated that BALP stayed in the stage of osteoblast and diminished ossification induced by Pb and Cd.

CTX was released in the process of bone resorption, as indicators of bone resorption. In proper conditions, a coupling exists between bone formation and resorption; an increase in the activity of bone resorption stimulates its formation [30]. Our result showed that the increased concentration of CTX in the serum after exposure to a mixture of Pb and Cd might be a consequence of the increased activity of osteoclasts. However, the enhanced resorption caused by Pb combined with Cd did not result in the stimulation of bone formation; the processes of bone turnover may remain uncoupled.

CT can inhibit the generation and activity of osteoclasts, resulting in the promoted deposi-

tion of bone calcium salt and lowering of blood calcium levels. PTH, as a potent stimulating factor for bone resorption, leads to Ca and Pi to be released into the blood. In this study, the serum concentration of CT decreased and the concentration of PTH increased. Pb induced disorders in mineral metabolism (especially Ca and Pi) because of the disruption of regulation through calciotropic hormones [8]. Cd exposure can cause the CT concentration to be depressed and the concentration of PTH enhanced, which leads to enhanced bone resorption in rats [10]. The disruption of the body status of Ca and Pi because of changes in the serum concentrations of calciotropic hormones noted in the rats exposed to Pb and Cd might induce bone resorption increase as well as disturb the mineral metabolism.

The Ca and Pi balance undergoes a strict hormonal regulation through CT and PTH, which regulates the concentration of Ca and Pi [10]. The skeleton is body storage of Ca and Pi, and in the case of the deficiency of these elements in the extracellular fluid, they are released from the bone to maintain calcemia and phosphatemia. Thus, disorders in the homeostasis of Ca and Pi are connected with changes in the bone mineral status [10]. Pb^{2+} ions easily displace Ca^{2+} ions in hydroxyapatites [31]. Pb can inhibit the absorption of Ca, leading to bone depression in rats [33]. Cd can inhibit the hydroxyapatite formation, resulting in bone mineral dissolution [12]. The increased urinary excretion of Ca has been reported to occur as a result of its decreased renal Ca^{2+} reabsorption induced by Cd [33]. Moreover, exposure to Cd can cause Ca deficiency, which leads to decreased Ca absorption or increased urinary loss [34]. The results of the present study demonstrated that the concentration of Ca and Pi in the serum decreased and the urinary Ca and Pi loss increased. The results showed that exposure to Pb and Cd can disorder Ca and Pi homeostasis and change their mineral status. Moreover, the decreased serum concentrations of Ca and Pi, simultaneous with the increased urinary loss of the two elements as a result of exposure to Pb and Cd, can be explained by their enhanced release from the skeleton because of the increased bone resorption.

In conclusion, the results of this study reveal that both Pb and Cd affect the bone metabolism in rats. The effects produced by the com-

bined treatment of metals not only increased the rate of bone turnover but also changed the concentration of calciotropic hormones, as well as caused disorders in the metabolism of Ca and Pi.

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Disclosure of conflict of interest

None.

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