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Low-exposure cadmium is more toxic on osteoporotic rat femoral bone: Mechanical, biochemical, and histopathological evaluation

Ulku Comelekoglu[a], Serap Yalin[b,*], Selda Bagis[c], Oya Ogenler[a], N. Ozlen Sahin[d], Altan Yildiz[e], Banu Coskun[f], Rezan Hatungil[g], Aysegul Turac[f]

[a]*Department of Biophysics, Mersin University Medical School, Mersin, Turkey*
[b]*Department of Biochemistry, Mersin University Pharmacy School, Mersin, Turkey*
[c]*Department of Physical Medicine and Rehabilitation, Mersin University Medical School, Mersin, Turkey*
[d]*Department of Pharmaceutical Technology, Mersin University Pharmacy School, Mersin, Turkey*
[e]*Department of Radiodiagnostics, Mersin University Medical School, Mersin, Turkey*
[f]*Department of Histology and Embryology, Mersin University Medical School, Mersin, Turkey*
[g]*Department of Physiology, Mersin University Medical School, Mersin, Turkey*

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Abstract

This study aimed to investigate the effects of low-exposure Cd on normal and osteoporotic bone. For this purpose, 12-week-old Sprague–Dawley female rats were assigned randomly to a control group, a Cd group, and an ovariectomy (OVX) + Cd group. OVX + Cd rats underwent bilateral ovariectomy via ventral incision. Twelve weeks after ovariectomy, cadmium chloride ($CdCl_2$) was given to rats (Cd and OVX + Cd groups) as intraperitoneal (ip) injection of 0.5 mg/kg three times a week for 18 weeks and distilled water was given to control group via ip route for 18 weeks. Bone mineral density (BMD) was measured at mid-diaphysis femoral region by dual-energy X-ray absorbsiometry. Cross-sectional area of the femoral shaft was evaluated by computerized tomography. Biomechanical measurements were performed at the mid-diaphysis of the left femur. Collagen fibers were evaluated at light microscopic level. BMD, cortical thickness, cortical area, and femur length were not changed in Cd and OVX + Cd groups in comparision to controls. In the OVX + Cd group, strength, displacement, energy, stress, strain, and toughness were significantly lower than those of the control group. The Cd concentration of bone was significantly increased in the OVX + Cd group compared to that in the control group. Collagen fiber intensity was decreased in all groups except control group. The results of the present study indicate that the administration of low-dose Cd does not affect normal bone biomechanical parameters, but it has a significant effect on osteoporotic bone.
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1. Introduction

Cadmium (Cd) is a toxic substance that is widely distributed in the environment and has a long biological half-life in organs. The kidneys, liver, bones, and respiratory and cardiovascular systems are the most important target organs for Cd toxicity (WHO, 1992) and Cd exposure can cause itai-itai disease, kidney tubular dysfunction, cancer, and bone damage (Freiberg et al., 1986a, b; Om et al., 2002; Itokawa et al., 1978). Reduced bone mineralization and increased risk of vertebral, hip, and forearm fractures have been reported after low to moderate exposure to Cd (Jarup et al., 1998; Staessen et al.,1999; Alfven et al., 2000; Wang et al.,2003; Brxoska and Moniuszko-Jakoniuk, 2004).

The mechanism of Cd-induced bone effects is not clear but several different possibilities have been suggested. Cd-induced bone effects may be mediated via renal tubular dysfunction (Berglund et al., 2000). The normal activation of vitamin D in the kidney also may be reduced and leads to decreased Ca absorption from the gut and impaired bone mineralization (Brzoska et al., 2005a). But recent studies including in vitro experiments carried out on bone

*Corresponding author. Fax: +90 324 341 30 22.
E-mail address: syalin01@hotmail.com (S. Yalin).

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cultures show that Cd acts directly on the activity and metabolism of bone cells and hydroxyapathyte formation (Iwami and Moriyama, 1993; Uriu et al., 2000).

Biomechanical integrity of bone is one of the most important factors related to bone strength and tendency to fracture. Bone ultimate strain, ultimate stress, stiffness, and toughness are predictor parameters for the evaluation of bone fragility and are measured by biomechanical test in this study. Although many studies have been conducted to clarify the mechanisms of Cd-induced bone damage, to our knowledge, only a few studies investigated the effects of cadmium on biomechanical parameters of bone (Brzoska et al., 2005a, b; Ogoshi et al., 1992). Also, the critical level of cadmium exposure that leads to fracture is still unknown. Brzoska et al. (2005a) reported that relatively low-level exposure of Cd (1 mg/L) caused an increase in fracture risk.

Cd is more accumulated and more toxic in growing skeleton than in mature skeleton. Disorders of bone properties that occur during skeletal growth at a young age will affect its properties in later life (Ogoshi et al., 1992; Hunder et al., 2001). Brzoska et al. (2005a) also showed that Cd-induced disturbances in Ca metabolism are closely related to changes in bone calcification. In addition, itai-itai disease is considered to be the most severe form of chronic Cd intoxication and 90% patients are postmenopausal women. Some authors (Jarup et al., 1998; Kjellstrom, 1992) suggested that estrogen depletion might play an important role in Cd toxication.

In the light of these studies, we suggested that cadmium might have a different effect on normal and osteoporotic bone and we designed this study to investigate this issue. Using biomechanical, biochemical, and histological methods, we investigated the effect of low-exposure Cd on normal and osteoporotic bone. The ovariectomized rat model was used to obtain the osteoporotic bone.

2. Material and methods

2.1. Animals

The Institutional Animal Care and Use Committee at Mersin University Medical Faculty approved the experiments described in this study. Twenty-one 12-week-old Sprague–Dawley female rats weighing 240–250 g each, obtained from Erciyes University Medical Faculty (Kayseri, Turkey), were assigned randomly to a control group ($n = 7$), Cd group ($n = 7$), and ovariectomy (OVX) + Cd group ($n = 7$). The animals were acclimatized for 1 week to our laboratory conditions prior to experimental manipulation. They had free access to standard laboratory chow and water ad libitum and were maintained on 12 h/12 h light–dark cycle. At the age of 13 weeks, OVX + Cd rats were anesthetized with ketamine (Ketalar; Eczacibasi Pharmaceutical Co.) and underwent bilateral ovariectomy via ventral incision. Twelve weeks after the ovariectomy, isotonic cadmium chloride solutions ($CdCl_2$) were given to rats (Cd and OVX + Cd groups) intraperitoneally (ip) three times a week at a dose of 0.5 mg/kg for 18 weeks. On the other side, distilled water was given to control group via ip route for 18 weeks.

Bone mineral density (BMD) was measured at mid-diaphysis femoral region by dual-energy X-ray absorbsiometry (DEXA; Norland 45 XR) adapted to the measurement of BMD in small animals. The length of the femur was measured with a digital clipper. Cross-sectional area of the femoral shaft was measured by computerized tomography (ARSTAR 40; Erlangen, Germany).

After 18 weeks of Cd administration, the study was terminated. At termination, blood was withdrawn from the heart for biochemical analysis and the bilateral femur of each animal was harvested. The left femur was stored at −20 °C until mechanical testing. The right femur was fixed with 10% neutral formalin and decalcified with 5% nitric acid solution for 1 day.

2.2. Biochemical analysis

Blood samples were collected (while rats were under anesthesia) from the heart and placed in tubes without anticoagulant. The samples were centrifuged within 30 min at 1500*g* for 5 min. Serum was separated and immediately frozen to −20 °C until analysis. Serum alkaline phosphatase, calcium and phosphorous levels, thyroid function tests, and parathormone, estrogen, progesterone, vitamin D_3, and magnesium levels were measured using an autoanalyzer in all groups.

2.3. Determination of metal concentration

Amounts of cadmium in bone were determined using Zeeman atomic absorption spectrophotometer 4100 ZL (Perkin Elmer) after sample preparation according to the method of Parker et al. (1967). Units are expressed as μg/g wet tissue for tissue metals.

2.4. Biomechanical test

Biomechanical measurements were performed at the mid-diaphysis of the left femur. Tensile test was performed to measure the maximum load, ultimate stress, ultimate strain stiffness, and toughness of bone. After thawing at room temperature, samples were tested using biomaterial testing machine (MAY 03; USA). For the tensile test, the femur bone was mounted horizontally in the machine by using colacryl. Distance between the two ends was 3 mm. The tensile loading speed in all tests was 1 mm/s. Data were transferred to the computers translating the numerical signals by 16-bit A/D converter for off line analysis. The sampling rate chosen was 1000 sample/s. During mounting and testing of the specimens, Ringer's solution was regularly applied for the prevention of drying. Load–displacement data were recorded using BIOPAC MP 100 Acquisition System Version 3.5.7 (Santa Barbara, USA). Ultimate tensile strength, stiffness, and displacement were determined from this curve. Ultimate tensile strength is the maximal load in tension that a material can sustain before failure (N). Displacement is the transverse displacement at the point of loading (mm). Stiffness was defined by the slope of the linear portion of the load–displacement curve (N/mm). These recordings were normalized by cross-sectional area and this curve was converted to a stress–strain curve. Stress–strain curves for each specimen were generated and the ultimate stress, ultimate strain, and toughness were determined. The stress (MPa) was calculated by dividing the failure load by the cross-sectional area of the specimen. The maximum strain was calculated as the displacement of the specimen divided by the initial gauge length (in mm/mm). The area under the stress–strain curve was defined as toughness, which is the energy required to cause breaking of the femoral shaft (MPa).

2.5. Histological evaluation

Specimens of right cortical bone were fixed with 10% neutral formalin and decalcified with 5% nitric acid solution for 1 day. The routine tissue processing for light microscopy was performed, and tissues were embedded in parafin. Cross-sections of 5-μm intervals were taken and stained with hematoxylin and eosin for measurement of cortical bone thickness and Masson Trichrom for collagen fiber evaluation. Diaphyseal cortical bone thickness was measured with ocular micrometer. Ten

random areas were selected and average thickness was calculated for each femur.

2.6. Statistical analysis

Statistical analysis was performed by using SPSS 10.0 software. Data were expressed as mean±SD at a significance level of $P<0.05$. After checking for normal distrubution and homogeneity of variances, the statistical analyses were carried out by ANOVA. Post hoc analysis of group differences was performed by LSD test.

3. Results

BMD, cortical thickness, cortical area, and femur length were unchanged in Cd group ($P = 0.205$, 0.754, 0.857, and 0.202, respectively) and OVX+Cd group ($P = 0.907$, 0.950, 0.747, and 0.177, respectively), in comparison to controls (Table 1). There were no statistically significant differences between the Cd and the OVX+Cd groups according to the BMD, cortical thickness, cortical area, and femur length ($P = 0.750$, 0.292, 0.128, and 0.670, respectively). The results of biomechanical tests are shown in Table 2. Strength, displacement, stiffness, energy, ultimate stress, ultimate strain, and toughness were reduced in Cd group but not significantly ($P = 0.258$, 0.277, 0.358, 0.192, 0.108, and 403, respectively). In the OVX+Cd group, strength, displacement, stiffness, energy, stress, strain, and toughness were significantly lower than those of the control group ($P = 0.047$, 0.03, 0.017, 0.05, 0.02, 0.035, and 0.009, respectively) and the Cd group ($P = 0.05$, 0.049, 0.038, 0.05, 0.042, 0.05, and 0.029, respectively). There were no statistically significant differences between the control, Cd, and OVX+Cd groups in serum calcium, phosphate, vitamin D_3, free T_3 (FT_3), free T_4 (FT_4), T_3, T_4, progesterone, and estrogen levels (Table 3). But the level of progesterone was significantly lower in the OVX+Cd group than in the control group ($P = 0.017$). The Cd concentration of bone was significantly increased in the OVX+Cd group compared to the control and Cd groups ($P = 0.027$ and 0.05, respectively) (Table 4). Masson trichrom stain (with light green) was applied to evaluate the collagen fibers at light microscopic level. Collagen fibers in compact bone were stained homogenously with light green in control group (Fig. 1). Alterations in the staining pattern of collagen fibers were observed in Cd and OVX+Cd groups. The staining intensity of the collagen fibers was prominently decreased in the Cd group (Fig. 2). In addition to this finding, collagen fiber staining was observed as red by Biebrich scarlett instead of light green in OVX+Cd group, which indicates that the structure of the collagen fibers may be damaged (Fig. 3).

Table 1
Geometric properties and bone mineral density of the diaphysial femur in control, Cd, and OVX+Cd groups of rats

Variables	Control	Cd	OVX+Cd
BMD (g/cm^2)	0.15±0.028	0.16±0.0019	0.15±0,013
Length (mm)	31.60±0.74	32.95±1.08	33.00±1.67
Area (mm^2)	8.1±0.94	8.3±0.67	8.50±1.09
Cortical thickness (mm)	0.45±0.0057	0.49±0.008	0.47±0.1

Table 2
Mechanical parameters of diaphysial femur in control, Cd, and OVX+Cd groups of rats

Variables	Control	Cd	OVX+Cd
Maximum load (N)	316.55±57.16	291.04±103.12	235.15±102.10[a,b]
Displacement (mm)	2.52±0.85	2.02±0.92	1.02±0.46[a,b]
Stiffness (N/mm)	139.85±57.16	198.50±59.27	377.33±98.58[a,b]
Energy (mJ)	393.03±135.72	304.99±168.65	184.65±47.93[a,b]
Stress (MPa)	25.55±3.63	19.00±7.73	17.35±4.84[a,b]
Strain	0.08±0.02	0.058±0.028	0.029±0.011[a,b]
Toughness (MPa)	0.82±0.35	0.513±0.28	0.23±0.02[a,b]

[a]Significant difference from control at $P<0.05$.
[b]Significant difference from Cd at $P<0.05$.

Table 3
Biochemical findings in control, Cd, and OVX+Cd groups of rats

Variables	Control	Cd	OVX+Cd
Calcium (mg/dL)	10.96±0.67	10.48±0.59	11.63±0.57
Phosphate (mg/dL)	9.28±1.57	8.70±1.52	8.76±1.25
Magnesium (mg/dL)	2.80±0.47	2.91±0.33	3.09±0.21
Alkaline phosphatase (U/L)	434±137.49	450±132.47	479.66±167.19
FT_3 (pmol/L)	4.89±0.43	4.67±1.00	5.31±1.14
FT_4 (pmol/L)	24.48±4.77	24.76±3.95	31.23±6.55
T_3 (ng/mL)	1.38±0.37	1.01±0.17	1.13±.20
T_4 (μg/dL)	5.19±0.74	4.41±0.88	5.65±1.13
Progesterone (ng/mL)	43.167±19.14	44.27±15.53	20.39±8.63[a]
Estrogen (pg/mL)	26.56±11.55	21.33±5.59	17.81±5.70
Vitamin D_3 (μg/L)	24.15±12.06	16.40±6.78	17.54±6.38

FT_3, free T_3 , FT_4, free T_4.
[a]Significant difference from control at $P<0.05$.

Table 4
Atomic absorbtion data of Cd concentration of bone in control, Cd, and OVX+Cd groups of rats

Groups	Cd concentration of bone (μg/g)
Control	$1.1\times10^{-2}\pm2.68\times10^{-3}$
Cd	$1.3\times10^{-2}\pm3.34\times10^{-3}$
OVX+Cd	$1.8\times10^{-2}\pm4.53\times10^{-3}$[a,b]

[a]Significant difference from control at $P<0.05$.
[b]Significant difference from Cd at $P<0.05$.

4. Discussion

In this study, we investigated the effect of cadmium exposure on normal and osteoporotic bone. It was found

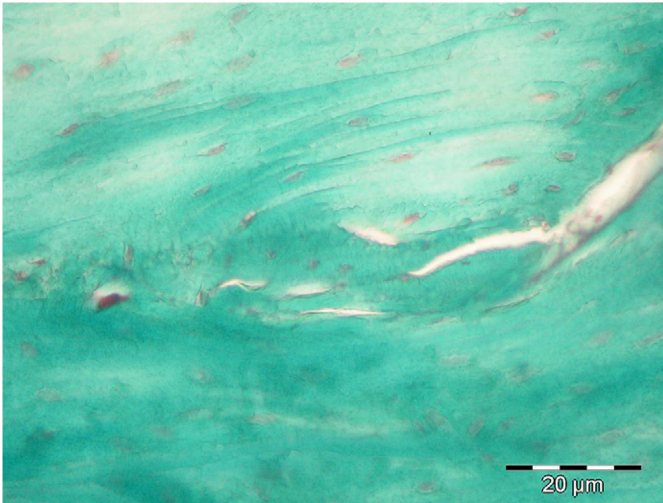

Fig. 1. Normal collagen fiber staining (Masson trichrom × 1200).

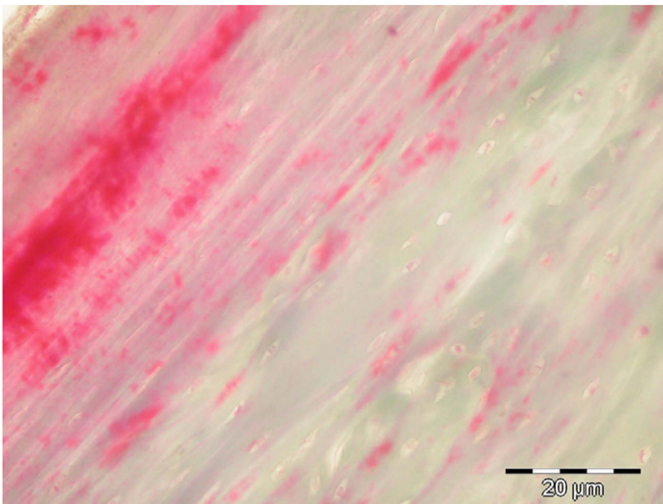

Fig. 2. The decrease in collagen fiber staining in the Cd group (Masson trichrom × 1200).

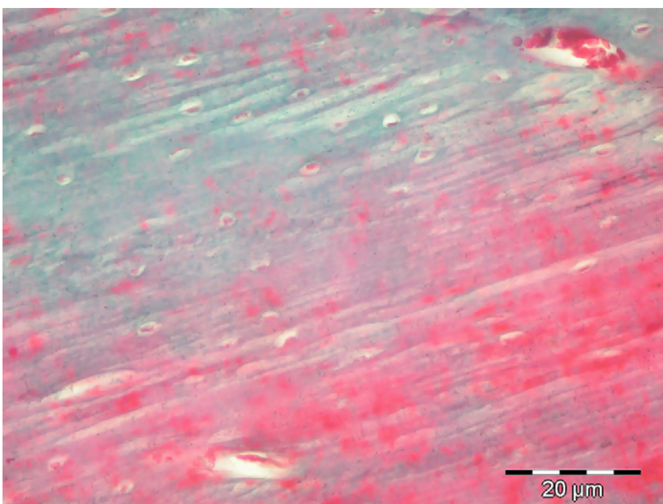

Fig. 3. The decrease in collagen fiber staining in the OVX + Cd group (Masson trichrom × 1200).

that cadmium is more toxic on osteoporotic bone. Bone biomechanic parameters and bone histology were more affected in osteoporotic rat than normal rat.

Some authors investigated the effect of cadmium on bone biomechanical properties and found that bone strength decreased even after low exposure to cadmium (Uriu et al., 2000; Brzoska et al., 2005a, b; Ogoshi et al., 1992). To our knowledge, there is no study investigating the effect of cadmium on normal and osteoporotic bone biomechanical parameters in detail.

In our study, cadmium exposure did not affect normal bone biomechanical parameters, but it showed a significant effect on osteoporotic bone. Significantly more increase in bone Cd content in OVX + Cd group than in control and Cd groups suggests that cadmium is more toxic on osteoporotic bone. The reason for such an effect was not clear. However, we assume that it may be related to the differences in bone metabolism and increased bone turnover in the postmenopausal period. Thus the calcium, phosphate, alkaline phosphatase, and vitamin D_3 levels were measured and no differences in the Cd and OVX + Cd groups were found in comparision to the control group. Furthermore, the thyroid and sex hormone levels were investigated in all groups, but no differences were found. Our results demonstrated that the effect of Cd is not related to mineral metabolism.

Some authors suggested that the decreasing bone strength was related to BMD (Jarup et al., 1998; Kjellstrom, 1992; Ogoshi et al., 1992). Recently, the NIH consensus reported that bone strength varies with BMD and bone quality. Bone quality varies with geometric properties (trabeculer network and macrostructure of the cortex and cortical shell) and material properties (matrix calcification and the composition and spatial arrangement of crystals, collagen fibers, and lamellae) (Ferretti et al., 2001; Martin and Boardman, 1993). Stiffness, strain, stress, and toughness were also important biomechanical parameters for bone strength. The increase in bone stiffness is not proportional to the increase in bone strength and BMD. The mineral component confers strength and stiffness to the tissues but, at increasing levels of stiffness, the tissue can become brittle, reducing the energy required for fracture. Strain is a change in bone length with ultimate stress and is related to bone strength and stiffness. Stress, strain, and toughness contribute to bone collagen integrity (Burr, 2002). We found that bone stress, strain, and toughness were reduced in the OVX + Cd group. The decrease in stress, strain, and toughness may be related to deformation of collagen integrity. Collagen fibers were evaluated by light microscopy and collagen fiber staining was decreased in Cd and OVX + Cd groups. But this reduction was more in the OVX + Cd group than in the Cd group. Galicka et al. (2004) reported that Cd influences collagen content and its solubility in the femoral bone of 3-week-old female rats. We thought that the collagen fiber structure may be damaged. Our biomechanical and histological results may suggest that any disturbances in

collagen metabolism results in the formation of low-quality bone tissue susceptible to deformation and fractures. The results of our study are consistent with those of Galicka et al. (2004).

In conclusion, the results obtained in this study show that the low dose of cadmium exposure did not significantly affect biomechanical parameters of normal healthy bone, but it has a significant effect on osteoporotic bone. It may be suggested that this effect may be related to collagen metabolism.

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