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Effects of Long-Term Administration of Clodronate on Growing Rat Bone

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Abstract. Bisphosphonates inhibit bone resorption. Short-term bisphosphonate treatment at therapeutical dosage has been shown to be safe, but there are only a few studies concerning the long-term effects of bisphosphonates on the non-osteoporotic skeleton. Here, we studied the bone effects of 32 weeks' treatment with clodronate on growing rats, using a therapeutical dose of 2 mg/kg and a high dose of 10 mg/kg. We used biomechanical, densitometrical, and, histomorphometrical analyses to examine the rat tibia, femur, and vertebra and also tested some hematological and biochemical parameters. Tibial length was significantly lower in the high clodronate group compared with the controls. No differences were found in tibial or vertebral ash weights. The L4 vertebra compression failure load was higher in the high clodronate group compared with the therapeutical clodronate group, but not compared with the controls. The mechanical strength of the femoral shaft or femoral neck was not affected by clodronate. Cortical BMD in the L4 vertebra was significantly higher in both clodronate groups compared with controls. Histomorphometrical analysis indicated that the trabecular number of vertebra was increased in the therapeutical clodronate group. The mineral apposition rate was not significantly affected by the treatment. Hematological analyses showed a decreased number of platelets at the high dosage. A slight increase in liver enzyme activity was seen in both groups. We conclude that long-term administration of clodronate has no harmful but rather some beneficial effects on bone at the therapeutical dosage. However, a fivefold dose of clodronate causes a slight decrease in the growth of tibial length.

Key words: Bisphosphonate — Clodronate — Long-term — Rat

Bisphosphonates decrease bone turnover and therefore bone loss [1]. They are widely used in the treatment of bone disorders associated with increased bone resorption, such as osteoporosis, Paget's disease, and metastatic bone diseases [2, 3].

Short-term treatments with therapeutical dosages of bisphosphonates have been shown to be safe in both experimental and clinical contexts [4, 5], however, etidronate at high doses has been shown to impair normal mineralization in animals as well as in human [4, 6], and impaired mineralization has been also reported after pamidronate treatment of Paget's disease and fibrous dysplasia [7]. The accumulation of bisphosphonate in human bone only reaches a plateau after years or even decades of administration [1]. It is also known that the skeletal half-life of bisphosphonates is long, between 3 months and 1 year [5]. It is therefore important to learn more about the long-term effects of clodronate.

There are only a few experimental studies of the long-term effect of bisphosphonates in skeleton. Etidronate has been shown to provoke spontaneous fractures and impaired normal mineralization in dogs after 12 months of treatment [6]. Beneficial bone effects of 2-year alendronate treatment in rats have been reported [8]. Moreover, 1-year tiludronate treatment administered to growing monkeys was safe [9], and 1-year pamindronate treatment of dogs increased bone stiffness, as calculated sonographically from the dog sterna [10]. In long-term studies of experimental osteoporosis, a 2-year treatment of baboons with alendronate [11] and zoledronate treatment of monkeys for 69 weeks prevented experimental osteopenia [12] and involved no adverse effects. There is only one study concerning the long-term effect of clodronate in the growing rat [13] in which it was found that a 6-month low dose of clodronate had beneficial effects on bone. No data on bone densitometry were available in that study.

The aim of the present study was to investigate the effects of long-term treatment of clodronate on the vertebrae, long bones, and blood chemistry in growing rats.

Material and Methods

Animals

A total of 100 3-month-old Sprague-Dawley rats with body weight of 228 (± 11.8) g were treated in three different ways for 32 weeks.

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The animals in group 1 received physiological saline (control), and the animals in group 2 were administered 2 mg/kg disodium clodronate (therapeutical clodronate) (Bonefos, Leiras Oy, Turku, Finland). The animals in group 3 were administered 10 mg/kg disodium clodronate (high clodronate). The lower dose (2 mg/kg) was chosen as a therapeutical dose, and the higher dose (10 mg/kg) was five times higher for safety purposes. All injections were given subcutaneously (s.c.) twice a week. The subcutaneous route was selected because absorption of clodronate after oral dosing is low. The right tibias were used in a fracture healing study to be presented elsewhere, and the left femurs and tibias as well as the vertebras were studied here.

The animals were fed *ad libitum* a special quality control (SQC) rat and mouse maintenance diet [RM1(E) SQC, Special Diets Services Limited, Witham, Essex, England] and allowed free access to tap water. The feed contained 0.71% calcium, 0.50% phosphorus, and 0.60 IU/g vitamin D_3. Food and water consumption was not determined. The rats were housed in individual cages at a constant temperature (21 ± 1.5°C) and relative humidity (30–65%) using a 12-hour light and darkness cycle (lights on at 7.00 a.m.). The experimental procedures were reviewed and approved by the Ethical Committee of Animal Experimentation in the local Provincial State Office of Western Finland.

For dynamic histomorphometry, each animal was given s.c. oxytetracycline 25 mg/kg (Terramycin® LA 200 mg/ml vet., Pfizer, Amboise, France) at the beginning of the study, eight animals per group received s.c. calcein 25 mg/kg (Sigma Chemical Co., St. Louis, USA) at 15 days, and xylenol orange 90 mg/kg (Sigma) at 8 days before the termination of the experiment.

After 32 weeks of administration, the animals were fasted overnight. Terminal blood samples were taken by cardiac puncture under anesthesia with CO_2 (CO_2: O_2 = 1:1) into Vacuette® tubes (gel and clot activator tubes, Greiner labortechnic, Austria). Serum was separated by centrifugation within 2 hours of sampling and divided into four test tubes: 2 × 0.2 ml, which were stored at −70°C, and the rest of the serum was divided into two tubes, which were stored at −20°C.

The following analyses were carried out from the blood samples at the end of the study. Hematocrit (B-HCR), hemoglobin concentration (B-Hb), total erythrocyte count (B-Eryt), mean cell volume (E-MCV), mean cell hemoglobin (E-MCH), mean cell hemoglobin concentration (E-MCHC), total leucocyte count (B-Leuc), and platelets (B-Plate) were measured using a Coulter T-540 analyzer (Coulter Electronics Ltd., Luton, England). The clinical chemistry variables analyzed from serum were alkaline phosphatase (S-ALP), aspartate aminotransferase (S-AST), alanine aminotransferase (S-ALT), total protein (S-Prot), albumin (S-Alb), bilirubin (S-Bil), and cholesterol (S-Chol), which were assessed using standard spectrophotometric methods. In addition, osteocalcin (S-OC) was measured by radioimmunoassay (Biomedical Technologies, Inc, MA, USA).

At necropsy, the tibias, femurs, and vertebras L1–L6 were dissected out. The bones (with the exception of those used for histomorphometry) were wrapped in saline-soaked gauze, stored in closed tubes at −20°, and thawed at room temperature on the day of analysis. Tibial length was measured using a vernier caliper.

For densitometry and a mechanical examination of the vertebrae, the spinous, transverse, and articulate processes of vertebra L4 were removed sharply with the standardized method, using a diamond saw. From the vertebral body, a 4.5-mm-high cylinder without endplates was obtained using a diamond saw with two parallel blades [13]. The bones were kept moistened during the densitometry and the mechanical examination.

Vertebrae L6 and L4 were carefully dissected out from eight animals/group and immersed in cold 40% ethanol. Thereafter, the vertebrae were transferred into 70% ethanol. The cylinders were processed for a histomorphometrical evaluation according to Schenk et al. [14]. Undecalcified sections (5–8 μm) were cut longitudinally from the vertebra L4 and transversally from the vertebra L6. The sections were stained by von Gossa/ toluidine blue [14]. In addition, one transversally cut unstained section from vertebra L6 was used for fluorescence-based dynamic histomorphometry. All examinations were performed blinded to the study groups.

Densitometry

The cylinder L4 and the femoral neck were scanned with a peripheral quantitative computer tomography (pQCT) system Stratec XCT 960A with the software version 5.20 (Norland Stratec Medizintechinik GmbH, Birkenfeld, Germany). The vertebral cylinders were scanned axially, with the anterior surface projected downward. A voxel size of 0.148 × 0.148 × 1.25 mm^3 was used. One cross-sectional slice was scanned at the middle of the specimen, as defined from the scout view of the pQCT system. An attenuation threshold value of 0.93 cm^{-1} (equivalent to 690 mg/cm^3) was used to measure the mean cortical bone mineral density (CtBMD) and the cross-sectional bone area (CSA). The mean trabecular mineral density (TrBMD) was measured using the peel mode 20. The trabecular area was set to 25% since a higher percentage resulted in partial inclusion of cortical bone in the area.

The femoral neck was scanned as previously described [15], with the femoral neck in the axial direction. The scan line was adjusted to mid-neck by using the scout view of the pQCT software. An attenuation threshold value of 0.93 cm^{-1} was used to measure the CtBMD and the CSA. Bone mineral content (BMC) was determined as well.

Biomechanical Testing

The vertebral L4 cylinder was mechanically tested as previously described [13]. The vertebral cylinder was positioned axially using a tilting support platen to reduce errors due to misalignment [16]. The bone was pressed axially at a constant compression speed of 0.155 mm/sec. The load curve was recorded by a plotter, and the bone failure load (Fmax) in compression was determined.

The femoral shaft was tested using a three-point bending method as described by Peng et al [17]. Briefly, the bone was compressed at the femoral midshaft using a constant compression speed of 0.155 mm/sec. A supporter with two loading points 13 mm apart was placed on the stage of the testing machine. The load curve was recorded, the bone failure load (Fmax) upon bending was determined, and stiffness was calculated as the slope of the linear part of the load-deformation curve.

Femoral neck was tested using axial compression as described previously [17]. Briefly, the proximal half of the femur was placed axially on a hole in a methylmetacrylate plate and pressed in a direction parallel to the femoral shaft at a constant compression speed of 0.155 mm/sec. The load curve was recorded and the femoral neck failure load (Fmax) was defined, and stiffness was calculated as the slope of the linear part of the load-deformation curve.

Ash Weight

The ash weight of the left tibia and vertebra L3 was determined after ashing at 600°C overnight.

Bone Histomorphometry

Static histomorphometrical analysis of vertebras L4 and L6 was carried out using a digital image analysis system (MCID/M4 with software version 3.0 rev. 1.1, Imaging Research Inc., Canada), a Sony color camera (DXC-930P Sony Co., Japan), and a Nikon Optiphot 2 microscope (Nikon, Japan) with a 1× objective (Nikon Plan 1/0.4 (160/–) at a pixel resolution of 0.014 × 0.015 mm. Standard histomorphometrical parameters were calculated [18, 19]. The unstained transversal body of vertebra L6 prelabeled with oxytetracycline, calcein, and xylenol orange was used for dynamic histomorphometry. The distance between the tetracycline and calcein labels of cortical bone and the distance between the calcein and xylenol orange labels of cancellous bone were measured using a confocal microscope (LSM 510 version 2.5, Germany) equipped with an Axiovert 100M inverted microscope and a 10× objective (Zeiss C-Apo/0.45W) at a pixel resolution of 1024 × 1024 pixels

Table 1. End body weight, ash weight of tibia and L3 vertebra, and length of tibia

Group	n	End body weight (g)	n	Ash weight tibia (mg)	Ash weight L3 vertebra (mg)	Length of tibia (mm)
Control	31	288 (14)	22–23	338 (22)	124 (13)	40.3 (0.4)
Clodronate (2 mg/kg)	32	283 (20)	22–24	343 (19)	124 (7)	40.0 (0.6)
Clodronate (10 mg/kg)	33	288 (16)	24–25	345 (17)	128 (11)	39.7 (0.6)[a]

Values are expressed as mean (SD)
[a] Clodronate vs control $P < 1.01$

(1.27 μm × 1.27 μm/pixel) and with fitc/rhodamine filters (BP 500–530 nm and LP 560 nm, Dicroic mirror 488/543 nm). The measurement was carried out on five to seven animals/group.

Statistical Analysis

All analyses were done with one-way ANOVA followed by the Scheffe post hoc test. If the distribution was not normal, the non-parametric Kruskal-Wallis, followed by the Mann Whitney U-test, was applied. A P value lower than 0.05 was considered statistically significant. The statistical analyses were carried out using the SPSS system (SPSS Inc. version 9.0.1, 1999). Numerical values are given as means and standard deviation (±SD).

Results

Body Weight, Tibia Length, Ash Weight

The animals tolerated the drug administration well. There were no significant differences in final body weights among the treatments (Table 1).

Tibias of the animals were slightly shorter in the high-clodronate group than in the control group (Table 1).

There were no significant differences in the ash weights of the L3 vertebras or the tibias among the different groups. (Table 1).

Biomechanical Testing

There were no significant differences in any mechanical parameters between the clodronate-treated animals and the controls. The vertebral compression failure load was higher in the high clodronate group when compared with the low clodronate group, but not when compared with controls. Femoral shaft or femoral neck strength was not affected by clodronate (Table 2).

Bone Densitometry

CtBMD in the L4 vertebral body was significantly greater in both clodronate groups compared with controls. There were no significant differences among the treatments in any other densitometrical parameters. (Table 3).

Histomorphometry

In longitudinal sections of the vertebra L4, the mean trabecular number was statistically significantly higher ($P < 0.05$) in the low clodronate group compared with controls but not in comparison with the high clodronate group. None of the other static histomorphometrical parameters showed any significant differences among the treatments (Table 4).

Mean values of the periosteal mineral apposition rate (MAR) in vertebrae L6 were 0.9 (±0.2) μm/day in the control group, 1.1 (±0.2) μm/day in the low-clodronate group, and 0.8 (±0.2) μm/day in the high clodronate group. The endosteal MAR of vertebra L6 was 1.8 (±0.2) μm/day, 2.0 (±0.4) μm/day, and 1.7 (±0.3) μm/day, respectively. There were no significant differences among the treatments in either the periosteal or endosteal MAR of vertebra L6.

Hematology and Clinical Chemistry

Clodronate appeared to have no significant effect on any of the hematological parameter at the therapeutical dose. The number of platelets was decreased ($P < 0.001$) and the mean cellular hemoglobin value was increased ($P < 0.05$) after the high clodronate treatment. (Table 5).

The aspartate aminotransferase and alanine aminotransferase activities were increased ($P < 0.01$) after both clodronate doses. The total proteins of serum were increased ($P < 0.05$) after the high clodronate treatment. Osteocalcin was decreased after both clodronate treatments ($P < 0.05$). There were no differences in albumin, bilirubin, cholesterol, or alkaline phosphatase activity (Table 6).

Discussion

Clodronate is typically used in situations where bone resorption is increased, such as tumor-induced osteolysis and osteoporosis [5]. Preclinical studies of bisphosphonates are also often carried out on animals with experimental osteoporosis [11, 12, 20, 21]. Here we studied the long-term effects of clodronate in growing non-osteoporotic rats.

Our results suggest that long-term administration of clodronate at therapeutic dosage has some beneficial effects and no adverse effects on the normal growing skeleton,

Table 2. Mechanical properties. Femoral shaft failure loads and stiffness, femoral neck failure load and stiffness, and L4 vertebra failure loads

		Femoral shaft		Femoral neck		L4
Group	n	Fmax (n)	Stiffness (n/mn)	Fmax (n)	Stiffness (n/mn)	Fmax (n)
Control	21–23	110 (8)	241 (63)	58 (7)	94 (15)	539 (101)
Clodronate 2 mg/kg	22–24	108 (10)	222 (41)	54 (6)	97 (14)	507 (62)
Clodronate 10 mg/kg	23–25	105 (9)	214 (29)	57 (7)	88 (19)	583 (103)[a]

Values are expressed as mean (SD).
[a] Clodronate 2 mg/kg vs clodronate 10 mg/kg $P < 0.05$

Table 3. Densitometry of vertebra L4 and femoral neck

		L4			Femoral neck		
Group	n	CSA (mm^2)	CtBMD (mg/cm^3)	TrBMD (mg/cm^3)	CSA (mm^2)	BMC (mg/mm)	CtBMD (mg/cm^3)
Control	23	7.0 (1.0)	1003 (36)	484 (65)	4.2 (0.4)	6.1 (0.6)	1180 (55)
Clodronate (2 mg/kg)	24	7.5 (0.9)	1035 (24)[a]	521 (68)	4.2 (0.4)	5.9 (0.5)	1174 (40)
Clodronate (10 mg/kg)	25	7.6 (0.9)	1033 (31)[a]	525 (68)	4.3 (0.5)	6.0 (0.6)	1194 (49)

Values are expressed as mean (SD)
[a] Clodronate vs control $P < 0.01$

Table 4. Histomorphometry. Longitudinal section of vertebra L4 and transversal section of vertebra L6, toluidine blue staining. Trabecular bone volume, trabecular thickness, trabecular number, trabecular separation, and trabecular bone pattern factor

	Longitudinal section of L4 vertebra						Transversal section of L6 vertebra		
Group	n	TBV (%)	Tb.Th (μm)	Tb.N (#/mm)	Tb.Sp (μm)	Tb.Pf(mm^{-1})	n	Ct.Ar (%)	Ct.Wi (μm)
Control	8	28 (4)	63 (8)	4.4 (0.3)	163 (18)	−5.8 (5.0)	8	23 (3)	119 (18)
Clodronate (2 mg/kg)	7	29 (7)	60 (10)	4.9 (0.3)[a]	147 (24)	−7.1 (3.5)	8	26 (7)	146 (34)
Clodronate 10 mg/kg	6	29 (6)	62 (8)	4.6 (0.4)	157 (31)	−6.1 (3.2)	8	22 (4)	158 (39)

Values are expressed as mean (SD)
TBV = Trabecular bone volume, Tb.Th = trabecular thickness, Tb.N = trabecular number, Tb.Sp = trabecular separation, Tb.Pf = trabecular bone pattern factor, Ct.Ar = cortical bone area, Ct.Wi = mean cortical width
[a] Clodronate vs control $P < 0.05$

which is in accordance with the previous studies with clodronate [13], tiludronate [9], pamidronate [10], and alendronate [8], but not with etidronate [6]. There was a slight decrease (1.5%) in tibial length at the clodronate dose fivefold compared with the therapeutic level, indicating a mild adverse effect on normal growth, but this change was not accompanied by changes in the mineral apposition rate or the mechanical properties. In the vertebra, cortical BMD was increased at the high dose of clodronate. Previously, long-term treatment with clodronate at high doses resulted in a decrease in the bone growth rate, which was not reflected in the mechanical quality of bone [13]. The rat femoral length was not affected by long-term alendronate treatment [8], and radial length in baboons was not affected by long-term tiludronate treatment [9]. Therefore, this decrease in bone length seems to relate solely to the high dose of clodronate.

Femoral neck has been prove to be a good indicator of different interventions affecting bone metabolism in rats and mouse [15, 17, 22]. In the present experiment, we found no influence of clodronate treatments on the femoral neck biomechanical properties. This was also supported by the previous findings obtained with clodronate [13].

Densitometry indicated a 3% increase in cortical BMD in vertebras after clodronate treatment. This change caused only a slight, statistically nonsignificant increment in ver-

Table 5. Hematology parameters

Group	n	B-Hb (g/l)	B-HCR (%)	B-Eryt (E12/l)	B-Leuc (E9/l)	B-Plate (E9/l)	E-MCV (fl)	E-MCH (pg)	E-MCHC (g/l)
Control	31	155 (5)	45.8 (1.8)	8.1 (0.3)	6.8 (2.2)	862 (135)	56.5 (1.6)	19.0 (0.6)	337 (5)
Clodronate (2 mg/kg)	32	155 (7)	45.8 (1.9)	8.1 (0.4)	6.5 (1.6)	836 (83)	56.9 (2.0)	19.2 (0.9)	338 (5)
Clodronate (10 mg/kg)	33	155 (7)	45.7 (2.1)	8.0 (0.4)	7.2 (1.6)	780 (95)[a]	57.3 (1.3)	19.5 (0.6)[b]	338 (4)

Values are expressed as mean (SD).
[a] Clodronate vs control $P < 0.001$
[b] Clodronate vs control $P < 0.01$

Table 6. Biochemical parameters

Group	n	S-AST (U/l)	S-ALP (U/l)	S-APS (U/l)	S-Bil (μmol/l)	S-Abl (g/l)	S-Prot (g/l)	S-Chol (mmol/l)	S-OC (μg/l)
Control	30	108 (27)	79 (20)	175 (49)	6.1 (0.7)	38.0 (3.9)	71.2 (4.3)	5.1 (0.9)	21.1 (5.2)
Clodronate (2 mg/kg)	32	134 (89)[b]	100 (45)[b]	186 (48)	6.1 (0.7)	37.2 (4.7)	72.4 (4.3)	5.5 (2.3)	19.6 (6.6)[c]
Clodronate (10 mg/kg)	33	137 (57)[b]	104 (22)[a]	170 (44)	6.3 (0.8)	74.4 (4.2)	74.4 (4.2)[c]	5.1 (1.3)	19.4 (6.7)[c]

Values are expressed as mean (SD).
[a] Clodronate vs control $P < 0.001$
[b] Clodronate vs control $P < 0.01$
[c] Clodronate vs control $P < 0.05$

tebral compression strength compared with controls. This is in good accordance with a previous study, in which long-term treatment with clodronate increased the compression strength of L4, but had no effect on the other biomechanical parameters [13].

Trabecular number (Tb.N) was increased at the therapeutical dose in vertebra, the effect of which was previously seen at a high dose of clodronate in tibia but not in vertebra [13]. This increment of Tb.N was accompanied by a tendency towards incremental trabecular BMD, which was not statistically significant.

At the therapeutical dosage, we found only a slight increase in the liver enzymes and a decrease in osteocalcin. Similar findings have previously been reported in a human study [23]. The treatment of clodronate had no significant hematological effect, although the high dose of clodronate decreased the number of platelets.

We conclude that long-term administration of clodronate has no harmful, but rather some beneficial effects on bone at the therapeutical dosage thus, it can be considered a safe medicine for osteoporosis. However, a fivefold dose of clodronate causes a slight decrease in the growth of tibial length.

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