Clodronate prevents immobilization osteopenia in rats

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We investigated the effect of clodronate on immobilization osteopenia (IO) induced by sciatic neurectomy in rats. 100 Wistar female rats were divided into 5 groups of 20 animals each: 1) sham-operated, control group, 2) IO + saline control group, 3) IO + clodronate 3 mg/kg/day, 4) IO + clodronate 10 mg/kg/day, and 5) IO + clodronate 30 mg/kg/day. Clodronate was administered subcutaneously beginning on the day after nerve sectioning. After 7 weeks, the animals were killed and both tibiae were removed. Bone mineral density, ash weight and calcium, phosphorus and magnesium contents of the ash of the tibiae were analyzed.

The weight of the rats did not differ between the groups during the experiment. The ash weight of the tibiae decreased by 6.6 percent and the mineral density decreased by 5.1 percent after neurectomy. Clodronate reduced IO in a dose-dependent manner and the highest dose neutralized the effect of neurectomy. The calcium content of the ash decreased after neurectomy as compared to the sham-operated group, and clodronate increased it to the sham-operated level. The bone Ca/P ratio remained normal.

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Bisphosphonates are a group of synthetic organic compounds that are structurally related to pyrophosphate and are known to be inhibitors of bone resorption. They are used to treat diseases in which bone resorption is increased, such as Paget's disease and hypercalcemia caused by malignancy (Fleisch 1989). Clodronate, one of the bisphosphonates, prevents immobilization osteopenia after resection of the second, third and fourth lumbar nerve trunks in rats (Fleisch et al. 1969, Mühlbauer et al. 1971) and after sciatic nerve sectioning in rats (Michael et al. 1971, Lane and Steinberg 1973) measured by ashing the bone.

We investigated the effect of clodronate on bone mineral density (BMD) in immobilization osteopenia (IO) induced by neurectomy in rats. The rat model of unilateral sciatic nerve section was selected, because it is simple to employ and gives a consistent reduction of bone-ash content of 9–11 percent over a period of one month (Lane and Steinberg 1973). The coherent/Compton scattering method has been shown to be suitable for trabecular bone mineral density measurements of human peripheral bone samples (Olkkonen et al. 1981). It has not been used previously on rat bones, but might offer a valuable tool for evaluating changes in BMD in immobilization osteopenia.

Animals and methods

100 Wistar female rats (20 weeks old, 244 (2) g, mean (*SEM*)) were used for the study, which was approved by a local animal ethics committee. The rats were housed in solid-bottom stainless steel cages (floor area 1029 cm^2) with aspen chip bedding (4HM, Finn-Tapvei, Finland), 4 rats in a cage. They were fed a pelleted rat diet (R3, Ewos-AB, Sweden) ad libitum and tap water was available. At the beginning of the study, the rats were divided into 5 groups with 20 animals in each:

I sham-operated, control group
II neurectomized + saline, control group
III neurect. + clodronate 3 mg/kg/day
IV neurect. + clodronate 10 mg/kg/day
V neurect. + clodronate 30 mg/kg/day

The rats were anesthetized with a combination of midazolam (Dormicum, Roche, Switzerland) and fentanyl-fluanisone (Hypnorm, Janssen Pharmaceutica, Belgium). Both drugs were diluted 1:1 using sterile water, and a syringe was filled with equal volumes of both dilutions. The anesthetic dose was 0.15–0.20 mL/100g, given intraperitoneally. A dorsolateral incision was made over the right hip and 1

cm of the sciatic nerve was resected. The contralateral leg was left intact.

Clodronate (Leiras Co, Finland) was dissolved in 0.9% saline and administered subcutaneously (sc) daily. We used the following solutions: 1.5 mg/mL, 5 mg/mL and 15 mg/mL to give doses of 3 mg/kg, 10 mg/kg and 30 mg/kg. Saline was administered to group II sc in the same volumes. Treatment was started 1 day after the operation and continued for 7 weeks. All animals were weighed weekly and dose levels were adjusted accordingly.

Metabolic cages were used for the 24-hour collection of urine from 12 rats in each group at the end of the study. The total volume of urine and the urinary excretions of calcium (U-Ca), creatinine (U-crea) and hydroxyproline (U-Hyp) were measured, employing routine laboratory methods. The urinary clodronate concentration was measured by a gas chromatographic method (Leiras Co, Turku, Finland) (Hanhijärvi et al. 1989). The percentage of clodronate excreted in the urine was calculated by dividing the amount in urine by the dose given sc. At the end of the study, the animals were anesthetized with premixed CO_2:O_2 (1:1) for cardiac puncture. Immediately thereafter, the rats were killed by cervical dislocation. Serum calcium (S-Ca), phosphate (S-Pi) and alkaline phosphatase (S-ALP) were measured using routine laboratory methods.

After the animals had been killed, the tibiae from both hind legs were dissected free from soft tissues. The fibula was detached from the tibia and discarded and the bone samples were placed in 70% alcohol.

The bone mineral density (BMD) of the proximal tibia was determined by a modification of the coherent/Compton scattering method (Olkkonen et al. 1981). The bone samples were irradiated by a narrow beam of 59.5 keV photons emitted from a 100 mCi ^{241}Am point source (Radiochemical Centre, Amersham, U.K.) equipped with a circular lead collimator (length 30 mm, diameter 6 mm). A Ge intrinsic semi-conductor detector (Ortec HP-56A) with a slit collimator (length 30 mm, slit dimensions 6 × 20mm) was used to measure the scattered radiation at an angle of 90°. The energy spectrum of the scattered photons was measured with a multichannel analyzer (Northern TN-1700). The intensity ratio of the coherent and Compton scattered photons was directly proportional to the average bone mineral density (g/cm^3) in the measuring site. A detailed calibration of the method is described elsewhere (Olkkonen et al.1981). The method was reproducible within 1.5 percent.

The tibiae were cut into 2 pieces and dried at 105 °C for 16 hours and then weighed. The dried bones were ashed at 600 °C for 16 hours and the ash was weighed. The ash was dissolved in 6 mL 3M HNO_3 and diluted to 50 mL with distilled water. The calcium and magnesium contents of the ash were determined using an atomic absorption spectrophotometer (Perkin Elmer, Model 372). Perkin Elmer Intensitron hollow cathode lamps were employed, and the operating parameters in the linear working range were as described in the manufacturer's operating manual. The phosphorus content of the ash was analyzed with a spectrophotometer (Hitachi, U-2000) (Fiske and Subbarow 1925).

The results are expressed as the mean *SEM*. The difference between right and left tibiae was evaluated by the Student's paired *t*-test and the difference between groups by analysis of variance (oneway, Student-Newman-Keuls). For Ca, Pi and Mg, the ash content was analyzed by the Kruskall-Wallis nonparametric test. A *P*-value less than 0.05 was considered significant. A statistical package for the social sciences (SPSS/PC+,V3.0, SPSS Inc., U.S.A.) was used for the statistical analyses.

Results

One rat in group III died after 1 week. All the other animals tolerated the experiment well. No differences in body weight were recorded between the groups. The weight of the rats increased slightly (11 *1* g) during 7 weeks.

The amount of urinary clodronate as a percentage of that given sc did not differ between the groups and was 17.3 *2.6*, 20.1 *3.3* and 17.3 *1.8* in clodronate groups 3, 10 and 30 mg. The effects of immobilization and of clodronate on biochemical parameters are shown in Table 1.

Sciatic neurectomy reduced both the dry (5.1 *0.8%*, $P < 0.001$) and ash weights (6.6 *0.8%*, $P < 0.001$) of the tibiae significantly when compared with control tibiae. Sham-operation did not influence the dry or ash weights. The ash weight loss in neurectomized tibiae was 2.1 *0.6%* (*P* 0.002), 1.8 *0.6%* (*P* 0.007), 0.5 *0.5%* (*P* 0.3) (Table 2) and dry weight loss 1.2 *0.5%* (*P* 0.03), 1.1 *0.6%* (*P* 0.06), 0.2 *0.6%* (*P* 0.7) in clodronate groups 3 mg, 10 mg and 30 mg, when compared with the control tibiae.

The amount of organic matrix of the tibiae, calculated by dry weight–ash weight, decreased only in the neurectomized control group (1.9 *0.9%*, *P* 0.05) when compared with control tibiae. Clodronate 3 mg increased the organic matrix of neurectomized tibiae when compared with the neurectomized control group ($P < 0.05$).

Table 1. Biochemical results (mean *SEM*)

Group	S-Ca (mmol/L)	S-Pi (mmol/L)	S-ALP (U/L)	U-HYP (µmol/L)	U-Ca/day (mmoL)	U-Ca/crea
I Sham	2.85 *0.02*	2.20 *0.04*	133 *11*	77 *5*	0.08 *0.01*	0.65 *0.05*
II Control IO + saline	2.92 *0.03*[a]	2.14 *0.05*	104 *6*	82 *9*	0.06 *0.01*	0.66 *0.05*
III IO + clodronate 3 mg/kg	2.79 *0.02*[bc]	1.96 *0.04*[ab]	121 *11*	68 *7*	0.06 *0.01*	0.51 *0.07*
IV IO + clodronate10 mg/kg	2.71 *0.03*[ab]	1.80 *0.06*[ab]	128 *13*	93 *6*	0.04 *0.00*[ab]	0.33 *0.03*[abd]
V IO + clodronate 30 mg/kg	2.84 *0.02*[bc]	1.85 *0.04*[ab]	147 *15*	76 *3*	0.03 *0.01*[abd]	0.31 *0.07*[abd]

[a]$P < 0.05$ compared with group I
[b]$P < 0.05$ compared with group II
IO neurectomized
[c]$P < 0.05$ compared with group IV
[d]$P < 0.05$ compared with group III
analysis of variance (Student-Newman-Keuls)

Table 2. Bone mineral density of the tibiae using coherent/Compton scattering method and ash weight of the tibia/weight of the rat (mean *SEM*)

Group	Bone mineral density (g/cm^3)		Ash weight/rat weight (percent)	
	Neurectomized (IO) or sham-operated tibiae	Control unoperated tibiae	Neurectomized (IO) or sham-operated tibiae	Control unoperated tibiae
I Sham	0.865 *0.007*	0.861 *0.007*	10.7 *0.2*	10.7 *0.1*
II Control IO + saline	0.797 *0.010*[ab]	0.840 *0.008*	10.1 *0.2*[ab]	10.8 *0.2*
III IO + clodronate 3 mg/kg	0.817 *0.005*[ab]	0.844 *0.006*	10.8 *0.2*[ac]	11.1 *0.2*
IV IO + clodronate10 mg/kg	0.828 *0.007*[abc]	0.845 *0.007*	10.8 *0.2*[ac]	11.0 *0.2*
V IO + clodronate 30 mg/kg	0.844 *0.007*[bcd]	0.849 *0.006*	10.9 *0.2*[c]	10.9 *0.2*

[a]$P < 0.05$ compared with control tibiae, paired t-test
[b]$P < 0.05$ compared with sham-operated tibiae, analysis of variance (Student-Newman-Keuls)
[c]$P < 0.05$ compared with neurectomized tibiae of control group, analysis of variance (Student-Newman-Keuls)
[d]$P < 0.05$ compared with neurectomized tibiae of clodronate 3 mg/kg group, analysis of variance (Student-Newman-Keuls)

Sham-operation did not influence the BMD. Neurectomy decreased the BMD markedly when compared with control tibiae (5.1 *1.0%*, $P < 0.001$). Clodronate increased the BMD in a dose-dependent manner and the difference between neurectomized and control tibiae was 3.1 *0.7%* ($P < 0.001$), 2.0 *0.7%* (P 0.01) and 0.6 *0.8%* (P 0.5) in clodronate groups 3 mg/kg, 10 mg/kg and 30 mg/kg/day (Table 2).

Immobilization reduced the calcium content of the ash of both tibiae in the neurectomized control group when compared with the sham-operated control group ($P < 0.05$), and the clodronate 30 mg/kg group had a higher ash calcium content than the neurectomized control group ($P < 0.05$). Neither neurectomy nor clodronate changed the calcium content of the ash significantly when compared with the control tibiae. There were no differences in ash phosphorus and magnesium contents between tibiae in the clodronate groups or between the clodronate groups and neurectomized or sham-operated groups. The ash Ca/P ratio did not differ between legs or groups.

Discussion

Immobilization reduced the ash weight of the tibia by 6.6 percent in our study. This agrees with the results of others after neurectomy in rats (Weinreb et al. 1989, Thompson et al. 1990). The mechanisms by which disuse is transduced to the cellular level are not known. During immobilization osteoclastic bone resorption is increased and osteoblastic bone formation decreased (Minaire et al. 1974, Weinreb et al. 1989). The same cellular events in immobilization osteopenia have been noticed in paraplegic patients, immobilized volunteers and immobilized animals (Minaire 1989).

Neurectomy reduced the calcium content of the ash in both operated and control tibiae to the same extent when compared with the sham-operated group. The loss of ash calcium in the control tibia may be due to a systemic effect. Thompson et al. (1990) found an increase in the number of osteoclasts of both neurectomized and control tibia after neurectomy, suggesting a systemic increase in the activation of resorption. The ash Ca/P ratio of the bone remained normal during the study in all groups.

Clodronate and other bisphosphonates are known to be inhibitors of bone resorption (Fleisch 1989), but they may also have direct effects on osteoblasts (Marie et al. 1985, Khokher and Dandona 1989). The mechanism by which bisphosphonates inhibit bone resorption is not known, but it is thought to be cellular (Fleisch 1989). 3 clodronate doses were chosen to test their effects on neurectomy-induced osteopenia. There were no differences in the body weights of the rats between groups and even after the highest dose of clodronate for 7 weeks the rats did not lose weight and there was no decrease in their activity level.

Immobilization osteopenia was prevented by clodronate in a dose-dependent manner. This result agrees with previous findings (Michael et al. 1971, Lane and Steinberg 1973). Ash weight of the immobilized tibia increased to the sham-operated level even after the lowest dose of clodronate and, with the dose of 10 mg/kg, there was a significant increase in BMD compared with the immobilized control group. The difference between legs was prevented only by the highest dose. Immobilization is known to increase bone resorption and our findings of increased bone ash weight, ash calcium content and BMD can be explained by the inhibition of bone resorption after neurectomy by clodronate.

After immobilization, S-Ca is usually normal or high–normal, but can be elevated. Serum phosphate values are in the high–normal range or markedly elevated and, after a spinal cord injury, the urine hydroxyproline excretion is known to increase (Minaire 1989). Mild hypercalcemia was observed in this study, but the phosphate concentration was unchanged when measured 7 weeks after neurectomy. Clodronate reduced serum calcium when compared with the immobilized group, an effect of clodronate also noted by others (McCloskey et al. 1987), but the hypocalcemic effect was the same in all doses used. The moderate degree of hypocalcemia produced by the administration of clodronate has been found to produce increased levels of PTH (McCloskey et al. 1987). The reduced serum phosphate values after clodronate can be explained by this effect of clodronate. The urinary excretion of calcium did not differ between sham-operated and neurectomy groups. Clodronate reduced urinary calcium excretion in accordance with the hypocalcemia produced by clodronate.

In conclusion, sciatic nerve sectioning caused immobilization-osteopenia and clodronate prevented it in a dose-dependent manner. The coherent/Compton scattering method is reliable in detecting BMD changes in the small bones of rats.

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