

# Clodronate reduces plate osteopenia in the rabbit

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An osteosynthesis with a four-hole AO/ASIF-DCP plate was performed on the right tibia of 40 rabbits. Clodronate (50 mg/kg s.c.) was given once a week, resulting in a mean bone concentration of 509 µg/g in 2 hours. Plate fixation caused a decrease in mean net cross-sectional area of compact cortical bone of 17 percent at 9 weeks and 46 percent at 18 weeks. This resulted from bone resorption in bone under the plate, from pronounced cavitation in the plated bone (about 5 percent of cortical bone area at 9 weeks and 15 percent at 18 weeks), and from the fact that the medullary space was increased by 15 percent at 18 weeks. The total cross-sectional area of the dia-

physis was increased by 31 percent at 9 weeks and by 17 percent at 18 weeks.

Clodronate treatment reduced cortical porosity to about half of the mean values in the placebo group. Clodronate increased both the calcium content in the retained bone and the cross-sectional area of compact cortical bone, but induced only an insignificant increase in the area of periosteal new bone. Clodronate treatment seems not to be contraindicated in conjunction with rigid osteosynthesis, and may even slow down the osteopenic response occurring under the rigid plate.

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Clodronate (dichloromethylene biphosphonate, Cl<sub>2</sub>MBP) is a second generation biphosphonate which hinders osteoclastic bone resorption. The effect is dose dependent and is mediated by cell damage and a reduction in the number of osteoclasts that excavate the bone surfaces upon which clodronate is first absorbed (Flanagan and Chambers 1989). Clodronate has no inhibitory effect on osteoid formation or osteoid mineralization (Bauer et al. 1986, Schenk et al. 1986, Nilsson et al. 1990). Biphosphonates reduce the bone loss resulting from immobilization (Cates et al. 1971, Michael et al. 1971).

In theory, the use of pharmaceutical agents to inhibit osteoclastic activity may be one way to minimize the harmful effects of rigid plate fixation. For this reason we wanted to evaluate whether clodronate therapy has any beneficial or untoward effects with respect to the osteopenia occurring during stress protection.

## Material and methods

40 male New Zealand white rabbits aged 11 months and weighing 2.7-4.7 kg were operated on under general anesthesia induced with Rombun vet 0.25 mL/kg

and Fentanyl 0.1 mL/kg s.c. A stainless steel (AISI 316L) four-hole dynamic plate (AO/ASIF-DCP) was applied to the anterolateral surface of the midshaft of the right tibia. The periosteum was left intact. The distal end of the plate was placed proximal to the tibiofibular junction. The plate was fixed with 4 cortical screws after drilling and pretapping of the screw-holes (Paavolainen et al. 1978, Slätis et al. 1978). The plate was 35 mm long, 7 mm wide and 1.5 mm thick. Neither a fracture nor an osteotomy on the bones was performed. An identical incision and closure on the left leg, leaving the left tibia intact, was performed as a sham operation. During the follow-up, the animals had free access to fully balanced food pellets (Evos K3, Tamro, Sweden).

Throughout the follow-up the animals were given clodronate subcutaneously, in a dose of 50 mg/kg, or only 0.9% NaCl injections. The first injection was given during the operation, and the last one week before the analysis.

To study matrix production in cortical bone, the animals were given 24 µCi <sup>3</sup>H-L-Proline (specific activity 100 Ci/mmol; Amersham, England) intramuscularly 2 days before analysis (Låftman et al. 1989). In the analysis, the hydroxyproline was extracted from a cortical bone sample, and the <sup>3</sup>H-proline activity was measured. The resultant specific activity of <sup>3</sup>H in the

extract was beneath the level that could have been measured reliably (less than 1-2 percent of the background level).

All skin incisions healed primarily, and no wound infections occurred. 2 of the 40 legs which underwent surgery were eliminated from the study because of a fissure in the bone through a screw-hole. One was a clodronate rabbit, eliminated at 9 weeks, and the other was a placebo rabbit, eliminated at 18 weeks.

After 9 or 18 weeks, the animals were exsanguinated under general anesthesia induced with barbiturate (Hypnorm). Serum was collected by centrifuging the blood sample; it was then stored at  $-70^{\circ}\text{C}$ . Serum alkaline phosphatase activity (U/L), calcium concentration (mmol/L) and inorganic phosphate (mmol/L) were determined colorimetrically with commercially available assay kits from Boehringer Mannheim GmbH (alkaline phosphatase and calcium) and Merck Diagnostica (inorganic phosphate).

The right and left tibiofibular bones were dissected out, with the periosteum intact. Anteroposterior and lateral radiographs (Agfa-Gevaert, 42 kV, 16 mAs, FFD 1 m) were taken of both tibiae. The plates were removed from the right tibiae, and the specimens were scrutinized macroscopically.

3 cross-sections were cut perpendicularly to the bone axis with an osteotome. The cross-sectional samples for histological and biochemical analyses were taken from the right tibiae at the midpoints between the 4 screw-holes beneath the plate, and from the left tibiae. The specimens were obtained from corresponding levels of each pair of tibiae by holding the right and left tibia together during cutting.

The specimens for histological analysis were fixed in buffered 10% formalin, and dehydrated in increasing concentrations of alcohol, defatted in xylene and embedded, but not decalcified, in methyl methacrylate and processed to methyl-methacrylate blocks by standard methods. During the hardening of the blocks, the osteotomized bone cylinders were laid on their osteotomized side on the bottom of block casts. 100- $\mu\text{m}$  sections were cut with a milling bone saw (Laitz 1600, Germany) perpendicular to the tibial axis. The calcified sections were stained by using a Masson-Goldner-Trichrome staining (Goldner 1938).

The cross-sectional areas of the different bone structures in the cross-sections were measured from the right and left tibiae with a computer-aided planimeter (Apple II with graphics table):

- 1) the area of compact cortical bone, excluding new bone formation;
- 2) the area of the medullary cavity;
- 3) the area of subperiosteal new bone (immature woven bone), which included both mineralized and unmineralized (osteoid) bone;

- 4) the total area of resorption cavities in compact cortical bone;
- 5) the percentage porosity, calculated from the proportion of the total area of resorption cavities to the area of compact cortical bone;
- 6) the area of the diaphysis including the medullary space, the compact cortical bone and the periosteal new bone.

The discrimination between compact cortical bone and subperiosteal new bone was made on the basis of their characteristic structures and color: compact cortical bone was stained darker green, and subperiosteal new bone included areas of osteoid, which were stained red.

Specimens reserved for chemical analyses were stored at  $-70^{\circ}\text{C}$ . The cross-sections of the diaphysis included both the compact cortical bone and the subperiosteal new bone around it. The specimens were weighed, cut into pieces and lyophilized to constant dry weight. The hydroxyproline, calcium, phosphorus, hexosamines, RNA, and DNA contents were determined (Penttinen 1972). The contents were expressed as the net amount (mg) and concentration (mg/g dry weight). The relation of RNA to DNA was calculated as an index of the capacity of cells to synthesize protein.

### Statistics

The multivariate analysis of variance was utilized to differentiate between the plated and control tibiae and between the clodronate and placebo specimens. No interaction was found between the grouping factors: the type of medication (clodronate or placebo), type of operation (plate fixation or no fixation), and the time of analysis. *P*-values over 0.05 were not considered significant.

## Results

The rigid plating resulted in radiographically clearly observable local osteopenia. By 18 weeks, the cortex under the plate was about 50 percent thinner than that of the contralateral leg. Examination of the radiographs revealed no macroscopic difference in the morphology of the tibiofibular bones between the clodronate-treated and placebo groups.

### Histologic evaluation

The typical remodeling changes related to rigid plating were evident after 9 weeks, and pronounced at 18

**Table 1. Histomorphometry of the plated and control tibiae in the clodronate-treated and placebo-treated rabbits at 9 and 18 weeks after operation. The areas (mm<sup>2</sup>) were measured on the cross-section of the diaphysis. Mean SD**

	9 weeks treatment		18 weeks treatment	
	Placebo	Clodronate	Placebo	Clodronate
<i>No of tibiae</i>				
Plated	10	9	9	10
Control	10	10	10	10
<i>Compact cortical bone area (mm<sup>2</sup>)</i>				
Plated	22.5 2.5	24.7 2.0	16.4 3.0 <sup>a</sup>	20.6 3.8 <sup>b</sup>
Control	25.7 3.4	27.2 3.2	26.1 3.1	27.2 5.3
<i>Subperiosteal new bone area (mm<sup>2</sup>)</i>				
Plated	14.5 4.9 <sup>a</sup>	16.2 8.3	14.6 3.2 <sup>a</sup>	17.9 8.8 <sup>c</sup>
Control	0 0	0 0	0 0	0 0
<i>Porosity in cortical bone (%)</i>				
Plated	5.2 6.4 <sup>a</sup>	1.8 1.4 <sup>c</sup>	15.1 13.8 <sup>d</sup>	7.7 7.0 <sup>c</sup>
Control	0.4 0.9	0.8 1.4	0.04 0.08	0.1 0.3
<i>Medullary space area (mm<sup>2</sup>)</i>				
Plated	10.7 3.4	8.4 1.4	14.2 3.2 <sup>a</sup>	13.9 4.1 <sup>c</sup>
Control	11.4 2.2	10.8 2.4	12.3 2.6	10.2 3.4

<sup>a</sup> Difference from control tibia,  $P < 0.05$   
<sup>b</sup> Difference from placebo group,  $P < 0.05$   
<sup>c</sup> No difference from placebo group,  $P > 0.05$   
<sup>d</sup> Difference from control tibia,  $P < 0.01$

weeks. Bone resorption had progressed along the endosteal surface of the cortex, and new bone had formed subperiosteally beside the plate and diametrically opposite to it. In consequence, the cortical width under the plate had decreased, and both the average marrow diameter and the average external diaphyseal diameter had increased (Table 1). The resorption cavities within the cortex were clearly increased in size.

#### Histomorphometric analysis (Table 1)

In the placebo group, the porosity in compact cortical bone increased to a mean value of 5 percent of cortical bone area at 9 weeks, and to 15 percent at 18 weeks. The corresponding mean value for the unplated tibiae was less than 1 percent, and the differences between plated and unplated tibiae were significant ( $P < 0.01$ ). In the clodronate-treated group, the porosity in cortical bone at 9 and 18 weeks was about half of that in the placebo group. Owing to the great interindividual variation, however, clodronate treatment could not be shown to induce a statistically significant improvement in porosity.

At 18 weeks, the area of medullary space in the plated compared to the unplated tibiae had increased by 15 percent ( $P < 0.05$ ). Clodronate therapy did not modify this.

**Table 2. Chemical analysis of the plated and control tibiae (mg/g dry weight) in the clodronate-treated and placebo-treated rabbits at 9 and 18 weeks after operation. Mean SD (n 10)**

	9 weeks treatment		18 weeks treatment	
	Placebo	Clodronate	Placebo	Clodronate
<i>Hexosamines content (mg/g dry weight)</i>				
Plated	1.30 0.19	1.41 0.19	1.13 0.08	1.18 0.10
Control	1.15 0.21	1.18 0.19	1.17 0.07	1.08 0.18
<i>DNA content (µg/g dry weight)</i>				
Plated	1.89 0.96	2.05 1.39	1.22 0.35	1.24 0.27
Control	2.20 1.05	2.16 1.27	0.72 0.13	1.02 0.36
<i>RNA to DNA ratio (%)</i>				
Plated	8.5 4.0	9.4 4.7	9.7 2.2	9.6 2.3
Control	6.6 24.9	8.0 3.7	16.0 2.8	12.3 4.0
<i>Calcium content (mg/g dry weight)</i>				
Plated	193 27	206 19	104 43	133 45 <sup>a</sup>
Control	189 14	197 23	101 15	127 30 <sup>a</sup>
<i>Phosphorus content (mg/g dry weight)</i>				
Plated	135 16	136 22	100 23	112 17
Control	128 28	129 20	106 16	113 18
<i>Hydroxyproline content (mg/g dry weight)</i>				
Plated	22.1 1.3	21.2 1.8	20.1 3.2	19.6 3.4
Control	19.3 1.7	19.8 2.0	16.3 3.3	17.7 3.6

No difference between the plated and control bones.

<sup>a</sup>Difference between clodronate group and placebo group at 18 weeks,  $P < 0.05$ .

In the placebo group, the area of compact cortical bone (excluding new bone formation) was smaller in the plated tibiae than in the unplated tibiae ( $P < 0.01$ ). Clodronate therapy caused a significant increase in the amount of compact cortical bone in the plated tibiae ( $P < 0.05$ ).

The amount of subperiosteal new bone (immature woven bone) was also significantly increased in plated tibiae ( $P < 0.001$ ). Furthermore, the area of new bone formation in the plated tibiae was somewhat, but not significantly, increased by clodronate.

As a result of the above changes the mean cross-sectional area of the plated tibiae (including the medullary space, the compact cortical bone and the periosteal new bone) was 31 percent greater at 9 weeks and 17 percent greater at 18 weeks ( $P < 0.01$ ) compared to unplated tibiae. The cross-sectional area of the diaphysis in plated tibiae was somewhat, but not significantly, increased by clodronate.

#### Chemical analysis

The chemical composition of bone did not differ between the plated and unplated tibiae.

The concentrations of RNA and DNA, reflecting the cell concentration in bone, and the RNA to DNA ratio, reflecting the capacity of cells to synthesize protein, were at the same level in the bone tissue in the

**Table 3.** The serum concentrations of calcium, inorganic phosphate (mmol/L) and the serum alkaline phosphatase activity (U/L) in the clodronate-treated and placebo-treated rabbits at 9 and 18 weeks after operation. Mean SD (n 10)

	9 weeks treatment		18 weeks treatment	
	Placebo	Clodronate	Placebo	Clodronate
<i>Serum calcium (mmol/L)</i>	3.6 0.2	3.5 0.2	3.6 0.2	3.4 0.2
<i>Serum phosphate (mmol/L)</i>	1.4 0.2	1.3 0.2	1.2 0.2	1.2 0.2
<i>Serum alkaline phosphorus activity (U/L)</i>	118 26	119 39	100 21	96 26

No difference between the clodronate and placebo groups

total cross-sections from plated tibiae in the clodronate-treated and placebo groups. Clodronate did not induce any significant changes in hexosamines (reflecting the amount of cartilage), hydroxyproline (reflecting the collagen content) or the phosphorus content in plated tibiae (Table 2).

The concentration of calcium in the diaphysis had decreased during the follow-up in both plated and unplated tibiae. The concentration of calcium in plated tibiae was higher in the clodronate-treated group ( $P < 0.01$ ).

#### *Chemical analysis of serum*

The concentrations of circulating calcium, phosphate and the activity of alkaline phosphatase were not changed by treatment with clodronate (Table 3).

## **Discussion**

The remodeling of bone under a rigid plate has been attributed to stress-shielding (Paavolainen et al. 1978, Woo et al. 1984) and to bone necrosis under the plate, irrespective of its rigidity (Vattolo 1986, Joerger 1987). The progression of porosity was rapid in our study. Furthermore, it was observed that the formation of cavitations in cortical bone connected with the formation of subperiosteal new bone greatly resembles the regional acceleratory phenomenon (RAP) of cortical bone (Frost 1989). RAP is known to be induced by bone injury, like a fracture or an osteotomy. It is known that under a plate there is a comparably large zone of disturbed blood supply to the bone (Perren et al. 1988). The appearance of RAP in the bone without fracture signifies local bone damage under the plate.

RAP is now considered a reparative response of bone to trauma, and must be distinguished from osteoporosis due to immobilization (Frost 1989). What is the role of osteoclastic inhibition in this setting?

Clodronate treatment slowed down the drastic remodeling of bone induced by the rigid plate; the volume of cortical compact diaphyseal bone was greater in clodronate-treated rabbits than in the placebo-treated animals. Furthermore, the increase in porosity induced by plate fixation may have been slowed down by clodronate, but the effect was not statistically significant. The increase in the volume of compact cortical plated bones in the clodronate animals is explained by the fact that clodronate specifically inhibits osteoclastic resorption of bone (Miller and Jee 1979, Schenk et al. 1986, Nilsson et al. 1990).

Clodronate treatment induced no significant increase in the formation of subperiosteal new bone in the plated tibiae, although the values detected in the treated animals were somewhat higher. In the clodronate animals, a slight increase in the area of subperiosteal new bone might have resulted from hampered remodeling of new bone; remodeling of subperiosteal new bone was not measured in the present study. The results indicate, however, that new bone formation can occur subperiosteally, independently of resorption activity, and that the mediator mechanisms involved in new bone formation are not inhibited by clodronate. Although it retards the osteoclastic function, clodronate has no effect on osteoid mineralization (Bauer et al. 1986, Schenk et al. 1986, Nilsson et al. 1990).

As expected, plating did not change the quality of the cortical bone matrix, when measured by chemical parameters (Paavolainen et al. 1978). It must be stressed that the contents of matrix components were measured in relation to the dry weight of bone, not to the volume. The reduced calcium concentration in diaphyseal bone detected both under the plate and in the contralateral unplated tibia during follow-up reflects the high turnover rate of bone, which was counterbalanced by clodronate treatment. Determination of the calcium concentration in bone does not make it possible to evaluate the rate of mineral formation and resorption. Unfortunately, the dynamic analysis of collagen turnover by tritiated proline labeling failed for technical reasons. Information on dynamic collagen formation and mineralization therefore was not obtained.

The kinetics of disodium clodronate in rabbits has been studied after subcutaneous administration of disodium clodronate, 50 mg/kg, once a week. High concentrations in serum were obtained 1 hour after subcutaneous administration. The levels declined rapidly thereafter, giving an estimated half-life of about 1 hour. A remarkable amount of the drug administered

(roughly over 60 percent) was found in bone 2 hours after the subcutaneous injection. The concentration in bone 2 hours after the subcutaneous injection was  $509.1 \pm 37.7 \mu\text{g/g}$  (Lauren and Österman 1990). Earlier it has been shown that high radioactivities in bone persist even 12 months after a single intravenous dose of  $^{14}\text{C}$ -clodronate (Mönkkönen 1988). The dosage and administration route of the drug were thus clearly adequate to reveal the anticipated effects.

We conclude that the early osteopenia occurring under a rigid plate and concomitant new bone formation may represent the regional acceleratory phenomenon. Clodronate treatment may be beneficial with respect to the osteopenic response occurring under rigid plate fixation. Subperiosteal new bone formation, reflecting the reparative response of bone, was not inhibited. Clodronate treatment thus seems not to be contraindicated in conjunction with rigid osteosynthesis, when it is indicated for other reasons.

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