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Comparison of incadronate and alfacalcidol on increased bone turnover caused by ovariectomy in rats

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Abstract

Mineral density of trabecular bone at the metaphyses of right tibiae was measured by peripheral quantitative computed tomography (pQCT) in ovariectomized rats. Bone mineral density (BMD) decreased dramatically in the 4 weeks following ovariectomy, suggesting that the method is sensitive enough to detect decreased bone mineral density within a short period. Orally administered incadronate dose dependently inhibited the decrease in trabecular bone mineral density induced by ovariectomy, as assessed 4 weeks after surgery. Significant inhibition was observed at doses of more than 0.3 mg/kg/day. Moreover, incadronate at doses of 1 mg/kg or more inhibited the increase in urinary deoxypyridinoline levels induced by ovariectomy, and although slightly increased serum intact parathyroid hormone (PTH) levels were observed, no significant alteration in serum calcium ion levels or urinary calcium excretion occurred. In contrast, while alfacalcidol inhibited the decrease in bone mineral density and the increase in urinary deoxypyridinoline levels at a dose of 300 ng/kg, it significantly lowered serum intact PTH levels and elevated serum free calcium levels as well as urinary calcium excretion. These results suggest that incadronate exerts its pharmacological effect (inhibition of bone resorption and increase in bone mass) by a mechanism different from that of alfacacidol.

Keywords: Bisphosphonate; Osteoporosis; Ovariectomy; Bone mineral density; Deoxypyridinoline

1. Introduction

Although the ovariectomized rat model (Kalu, 1991) is widely used to assess candidate drugs for the treatment of osteoporosis, several months are generally needed to evaluate changes in bone mineral density. Recent reports indicate that the loss of the trabecular bone mass is more marked than the loss of cortical bone mass (Omi and Ezawa, 1995). Furthermore, peripheral quantitative computed tomography (pQCT) has been developed to measure specifically the change in trabecular bone mineral density (BMD), and this method provides the possibility to determine the effect of drugs on the change in BMD more accurately and earlier, at 4 weeks (Tsurusaki et al., 2000). Therefore, we also used the pQCT method to determine trabecular BMD at bone metaphyses of the right tibiae.

Etidronate, one of the first-generation bisphosphonates, caused the mineralization of bone after long-term administration as a major side effect. In contrast, new bisphosphonates, categorized as the third-generation bisphosphonates, have no effect on the delay in mineralization and exert potent effects on bone resorption. When we compared the effect of incadronate on BMD in ovariectomized rats with that of etidronate and alendronate, we found that incadronate was 240-fold more potent than etidronate and 4-fold more potent than alendronate (Iwai et al., in preparation). In this paper, incadronate (Abe et al., 1990; Kawamuki et al., 1990; Kudo et al., 1990; Fujimoto et al., 1990; Tanaka et al., 1995; Motoie et al., 1996; Takahashi et al., 1998) was administered to ovariectomized rats orally for 4 weeks and the effects of the drug on urinary deoxypyridinoline, as a bone turnover maker, and on tibial trabecular BMD were assessed using the pQCT method and compared with those of alfacalcidol [1α(OH)vitamin D_3] (Shiraki et al., 1993, 1996; Menczel

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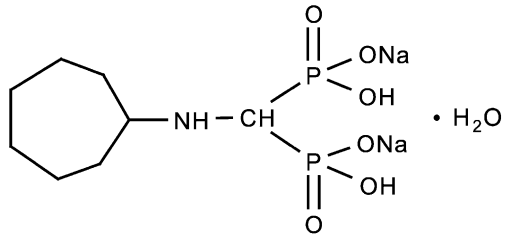

Fig. 1. Chemical structure of incadronate disodium monohydrate.

et al., 1994; Orimo et al., 1994; Chen et al., 1997), which is widely used to treat osteoporosis in Japan.

2. Materials and methods

2.1. Materials and methods

Incadronate disodium monohydrate (Fig. 1) was synthesized by Yamanouchi Pharmaceutical (Takeuchi et al., 1993) and was dissolved in distilled water at 5 ml/kg and then administered orally as an aqueous solution. Alfacalcidol was purchased from Wako (Osaka, Japan). It was first dissolved in a small amount of ethanol and stored −40 °C. Dosing solutions were prepared in corn oil to yield dose volumes of 5 ml/kg immediately before administration.

Mature female Sprague–Dawley rats (12 weeks), purchased from Charles River Japan (Kanagawa, Japan), were allowed free access to tap water and commercial standard solid food containing 1.18% calcium and 2.5 IU/g of vitamin D_3 (CE-2; CLEA Japan, Tokyo, Japan). After 7 days of acclimatization, all animals were anesthetized with pentobarbital (40 mg/kg, i.p.), and their both ovaries were exposed. Immediately, the ovaries of all animals, except those of the sham-operated group, were ligated and then removed. Sham operation was performed by only exposing the ovaries. From the day after ovariectomy, either incadronate (0.1, 0.3, 1, 3, 10 mg/kg) or alfacalcidol (30, 100, 300 ng/kg) was administered orally 6 times a week for 4 weeks. After the final administration, urine (0–24 h) was collected to assay urinary deoxypyridinoline levels by high-performance liquid chromatography in Teijin Bio Laboratories (Tokyo, Japan). Urinary calcium excretion was measured by MXB method using Calcium E test WAKO kit purchased from Wako and urinary creatine levels were measured by auto-analyzer in Teijin Bio Laboratories. Rats were anesthetized and then killed by exsanguination from the abdominal aorta. Blood was collected in vacuum sampling tubes VT070B (Terumo, Tokyo, Japan) through a laparotomic incision. Serum samples were prepared by centrifugation (1000 × *g* for 20 min); the samples were used to determine serum free calcium levels, using an electrolyte analyzer SERA252 (Horiba, Kyoto, Japan), and serum intact parathyroid hormone (PTH) levels by radioimmunoassay in

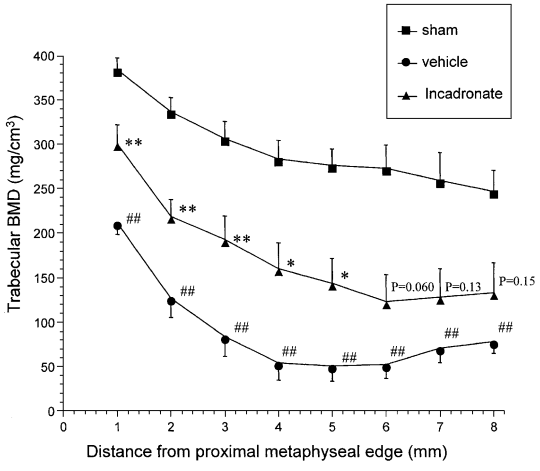

Fig. 2. Effect of incadronate on trabecular bone mineral density (BMD) of tibiae in ovariectomized rats. Each point and bar represents the mean ± S.E.M. ($n = 7$); #: statistical significance from each point of the sham control group; (##$P < 0.01$, Student's *t*-test); *: statistical significance from each point of the vehicle control group (* $P < 0.05$, ** $P < 0.01$, Student's *t*-test). The trabecular BMD of right tibial metaphyses were measured laminagraphically using 1-mm sections of the metaphysis scanning in a proximal to distal direction. BMD values in each 1-mm scan section markedly decreased following ovariectomy. Animals given 1 mg/kg p.o. incadronate had a significantly lower trabecular BMD.

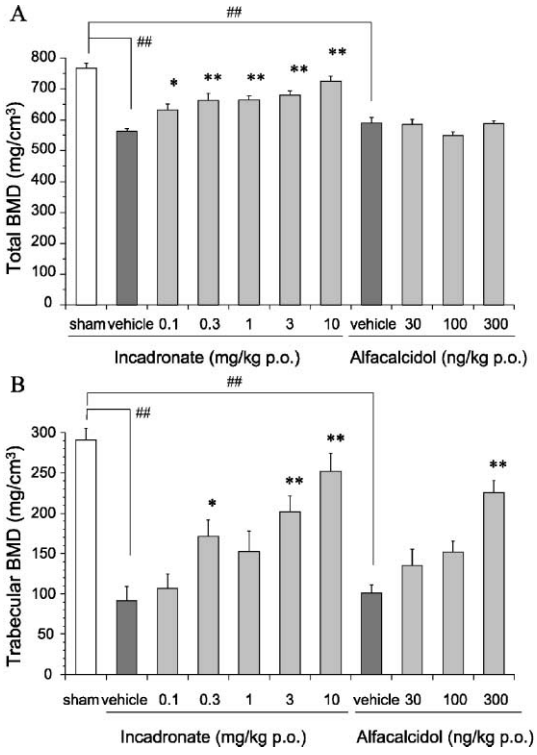

Fig. 3. Effects of incadronate and alfacalcidol on total and trabecular bone mineral density (BMD) of tibiae in ovariectomized rats. Each column and bar represents the mean ± S.E.M. ($n = 9–10$); #: statistical significance from the sham control group; (##$P < 0.01$, Student's *t*-test); *: statistical significance from the vehicle control group (* $P < 0.05$, ** $P < 0.01$, Dunnett's multiple range test). The total BMD (A) and trabecular BMD (B) were measured 3 mm from the edge of the proximal metaphysis. These results indicated that monitoring the change in trabecular BMD is more sensitive than measuring the change in total BMD.

Mitsubishi Kagaku Bio-Clinical Laboratories (Tokyo, Japan). Immediately after collection of urine and blood from rats, the right tibia was removed from each animal. The total and trabecular BMD of right tibial metaphyses were measured laminagraphically using 1-mm sections of the metaphysis scanned in a proximal to distal direction by an XCT-960A (Norland-Stratec, Waltham, MA, USA). The trabecular BMD 3 mm from the edge of the proximal metaphysis was used to evaluate the effect of the compounds.

2.2. Statistical analysis

Student's *t*-test was used to compare data between two groups, and Dunnett's multiple range test was used for more than two groups. A P value less than 0.05 was treated as statistically significant. Data are expressed as means ± S.E.M.

3. Results

Trabecular BMD results are shown in Fig. 2. BMD in each 1-mm scan section decreased 45–82% following ovariectomy, the most marked change appearing 3–6 mm from the proximal metaphyseal edge. Trabecular BMD of animals given 1 mg/kg incadronate was 31–52% greater than in animals receiving no treatment. Total BMD 3 mm from the proximal metaphyseal edge 4 weeks after surgery was 767 mg/cm^3 in animals which received the sham operation, 563 mg/cm^3 in ovariectomized animals treated with incadronate vehicle for 4 weeks, and 590 mg/cm^3 in animals treated with alfacalcidol vehicle. In contrast, the trabecular BMD at this site was 291 mg/cm^3 in the sham-operated group, and in the incadronate and alfacalcidol vehicle control groups it dramatically declined to 92.0 and

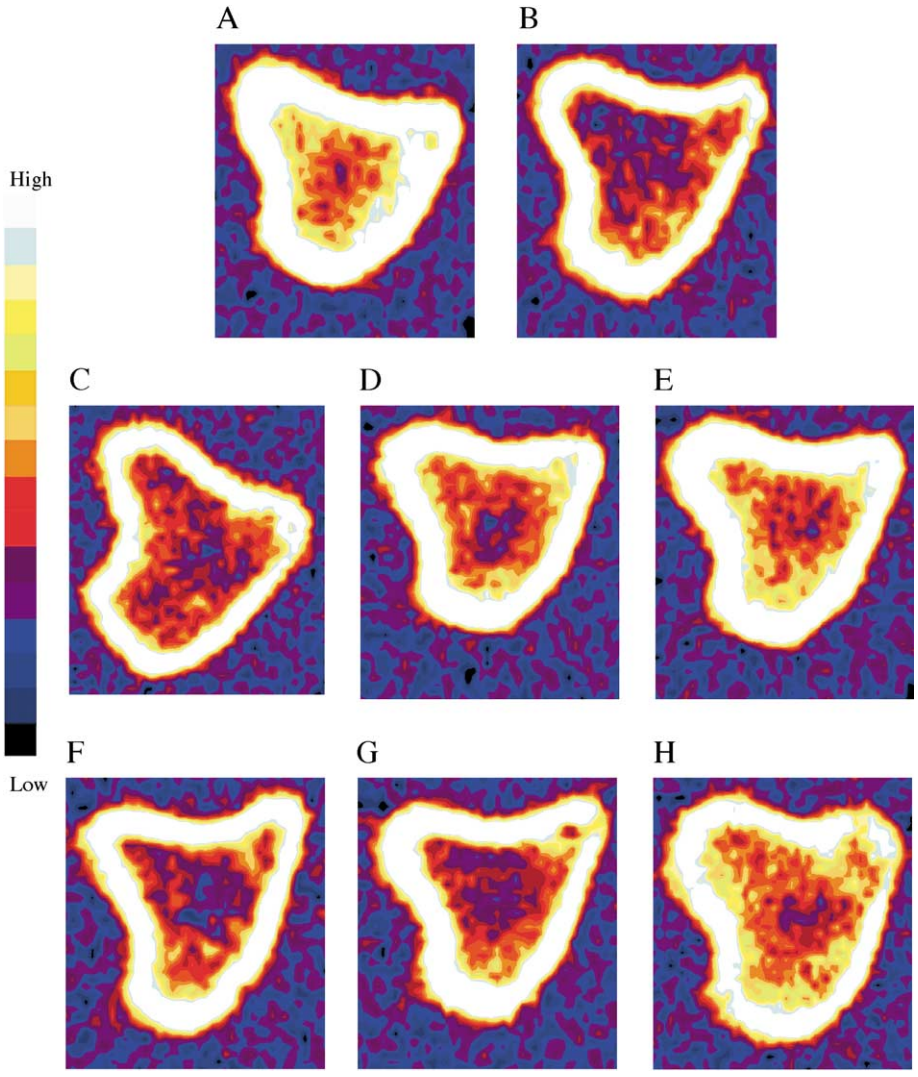

Fig. 4. Effects of incadronate and alfacalcidol on pQCT tomograms of tibiae in ovariectomized rats. (A) Sham operation, (B) incadronate vehicle, (C) Incadronate 0.1 mg/kg p.o., (D) incadronate 1 mg/kg p.o., (E) incadronate 10 mg/kg p.o., (F) alfacalcidol vehicle, (G) alfacalcidol 30 ng/kg, p.o., (H) alfacalcidol 300 ng/kg p.o. These representative tomograms show BMD by color brightness. Color brightness of each vehicle group was darker than that of the sham-operated group, showing lower BMD. Administration of each compound led to a change in the color from dark to bright, approaching that of the sham-operated group.

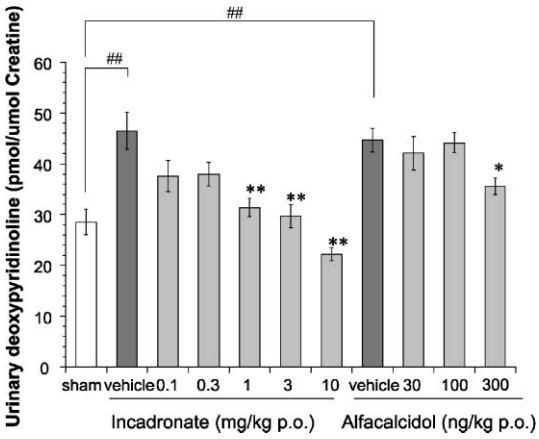

Fig. 5. Effects of incadronate and alfacalcidol on urinary deoxypyridinoline levels in ovariectomized rats. Each column and bar represents the mean ± S.E.M. ($n = 9–10$); #: statistical significance from the sham control group; (##$P < 0.01$, Student's t-test); *: statistical significance from the vehicle control group (*$P < 0.05$, **$P < 0.01$, Dunnett's multiple range test). Urinary deoxypyridinoline levels were measured by HPLC. At high doses of both compounds, urinary deoxypyridinoline levels remained low.

101 mg/cm^3, respectively, 4 weeks after ovariectomy (Figs. 3 and 4).

Ovariectomy significantly increased urinary deoxypyridinoline levels by 63% in the incadronate vehicle group and by 57% in the alfacalcidol vehicle group 4 weeks after surgery (Fig. 5). However, ovariectomy had no significant effect on serum free calcium levels or urinary calcium excretion (Table 1).

Oral administration of incadronate significantly and dose dependently retarded the BMD decrease caused by ovariectomy. Significant inhibition was observed at incadronate doses of more than 0.3 mg/kg; at a dose of 10 mg/kg, almost complete inhibition was observed. Incadronate also dose dependently lowered urinary deoxypyridinoline levels induced by ovariectomy, with significant inhibition at doses of more than 1 mg/kg (Fig. 5). Although incadronate administration caused a slight increase in PTH levels, it had no significant effects on serum free calcium levels or urinary calcium levels (Table 1). In contrast, although 300 ng/kg alfacalcidol also partially retarded both the loss of trabecular BMD (Fig. 3) and the increase in urinary deoxypyridinoline levels (Fig. 5), it significantly lowered serum intact PTH levels and significantly increased both serum free calcium levels and urinary calcium excretion (Table 1).

4. Discussion

Bisphosphonates are derivatives of pyrophosphates, possessing a P−C−P bond. Since this particular structure exhibits high affinity for hydroxyapatite crystals and distributes selectively in bone tissue, bisphosphonate have proved to be inhibitors of bone resorption. It is reported that some of the bisphosphonates cause functional disorders in osteoclasts, possibly caused by osteoclast apoptosis, which prevents osteoclasts from resorping bone (Hughes et al., 1995; Selander et al., 1996). Incadronate is a novel bisphosphonate and acts as a potent inhibitor of bone resorption (Abe et al., 1990; Kawamuki et al., 1990; Kudo et al., 1990; Fujimoto et al., 1990; Takeuchi et al., 1993). The drug thus has great potential for the treatment of various diseases caused by increased bone resorption, such as hypercalcemia (Takahashi et al., 1998) and osteoporosis (Motoie et al., 1995, 1996).

The results showed that there is a good relation between the decrease in urinary deoxypyridinoline levels and the reduction in BMD, while there is virtually no change in systemic calcium metabolism, except for the slight increase in PTH, which is considered to reflect compensatory secretion against the decrease in serum calcium levels following the inhibition of bone resorption. This slight elevation of PTH values was seen in another experiment (data not

Table 1
Effects of incadronate and alfacalcidol on systemic calcium metabolism in ovariectomized rats

Compound	Dose	Serum intact PTH (pg/ml)	Serum Ca^{2+} (mM)	Urinary Ca^{2+} excretion (mg/mg creatine)
Sham-operated	–	7.32 ± 0.40	1.54 ± 0.02	0.0782 ± 0.0185
Vehicle	0	7.95 ± 0.73	1.52 ± 0.01	0.0939 ± 0.0178
Incadronate	0.1	11.34 ± 1.14	1.53 ± 0.01	0.0939 ± 0.0216
(mg/kg p.o.)	0.3	8.13 ± 0.58	1.55 ± 0.02	0.1428 ± 0.0274
	1	22.71 ± 7.53[a]	1.48 ± 0.02	0.0927 ± 0.0188
	3	14.45 ± 2.36	1.49 ± 0.02	0.0988 ± 0.0142
	10	16.98 ± 4.29	1.49 ± 0.01	0.1098 ± 0.0269
Vehicle	0	10.28 ± 1.08[b]	1.50 ± 0.02	0.0870 ± 0.0088
Alfacalcidol	30	10.53 ± 2.34	1.52 ± 0.01	0.1123 ± 0.0180
(ng/kg p.o.)	100	7.64 ± 0.85	1.53 ± 0.01	0.1621 ± 0.0163[a]
	300	3.48 ± 0.23[c]	1.66 ± 0.03[c]	0.2709 ± 0.0269[c]

Each column and bar represents the mean ± S.E.M. ($n = 9–10$).
Three parameters of systemic calcium metabolism in rat serum and urine were determined. At a dose of 300 ng/kg, alfacalcidol significantly lowered serum intact PTH levels. It also significantly increased both serum free calcium levels and urinary calcium excretion.
[a] Statistical significance from the vehicle group ($P < 0.05$, Dunnett's multiple range test).
[b] Statistical significance from the sham control group ($P < 0.05$, Student's t-test).
[c] Statistical significance from the vehicle group ($P < 0.01$, Dunnett's multiple range test).

shown), and was also found in the paper about alendronate (Greenspan et al., 1996). This trend, however, is not always observed, suggesting that PTH levels may increase under certain conditions only. These findings support that the inhibitory effect of incadronate against the decrease in BMD can be predominantly attributed to the direct inhibition of bone resorption by the drug.

Alfacalcidol is a synthetic precursor of $1,25(OH)_2D_3$ and is converted into the active form in the liver, increasing intestinal calcium absorption (Raisz et al., 1972) and suppressing PTH (Chapuy et al., 1992; Ooms et al., 1995; Sherwood and Russel, 1989; Slatopolsky et al., 1984). Although $1,25(OH)_2D_3$ is also known as a bone resorption factor (Raisz et al., 1972), alfacalcidol is used as a therapeutic agent to increase BMD (Caniggia et al., 1990), to increase bone volume (Gallagher et al., 1982), and to decrease the incidence of hip fractures in senile osteoporotic patients (Gallagher et al., 1989; Tilyard et al., 1992). It remains unclear whether alfacalcidol changes bone resorption markers or not (Ooms et al., 1995; Cosman et al., 1995; Duda et al., 1987).

In the present study, alfacalcidol at a dose of 300 ng/kg significantly increased BMD and decreased urinary deoxypyridinoline levels, a bone resorption makers, suggesting that alfacalcidol decreases PTH and then indirectly reduces bone resorption, and consequently leading to an increase in bone volume.

One of the mechanisms for the inhibition of serum PTH levels would be the direct inhibition of PTH secretion by $1,25(OH)_2D_3$ converted from alfacalcidol. The indirect inhibition of PTH secretion would be considerable when the serum free calcium levels are increased, followed by an increase in calcium resorption from the gastrointestinal tract by $1,25(OH)_2D_3$.

Although the above-mentioned results are consistent with the clinical data obtained by Chen et al. (1997), the animal model used in our laboratories yielded contradictory results in that alfacalcidol administration did not alter the serum calcitonin levels or urinary phosphorus levels (data not shown).

It is therefore considered that alfacalcidol inhibits the inhibition of bone resorption and decreases BMD by an indirect mechanism through systemic regulation of calcium metabolism.

In the present study, the total BMD of the vehicle control group was lower than that of the sham-operated group (4 weeks after ovariectomy) by 23–27%. In contrast, trabecular BMD of vehicle-treated animals was 65–68% lower than that of the sham-operated animals, indicating that the change in trabecular BMD is 2.6–2.8 times greater than that in total BMD (Fig. 3A,B). These results demonstrate that trabecular bone regions are sensitive enough to evaluate changes in BMD. Furthermore, in the metaphysis evaluated with pQCT, 4 weeks of administration was enough to reveal decreases in BMD, while the density of whole bone measured with dual energy X-ray absorptiometry required several months of treatment to detect a significant change in BMD (data not shown). In this study, second lumbar vertebrae were used to measure whole BMD by dual energy X-ray absorptiometry 4 weeks after ovariectomy, and revealed that BMD 4 weeks after surgery was 0.2212 ± 0.0027 g/cm^2 in sham-operated rats, while that of control rats in the incadronate and alfacalcidol group was 0.2117 ± 0.0031 and 0.2169 ± 0.0023 g/cm^2, respectively. The value of probability (P value) against sham operation was as high as 0.031 and 0.235, respectively. In contrast, all of the P values obtained from the trabecular and total tibial BMD measurements with pQCT were 0. These results clearly demonstrate that the sensitivity of the pQCT method is high enough to evaluate BMD. Four weeks after ovariectomy, total BMD in the vehicle control group were significantly lower than those in sham-operated group. Incadronate inhibited this decrease significantly. Alfacalcidol, however, had no effect on total BMD. Furthermore, incadronate had the opposite effect to alfacalcidol on the PTH level, that is, this was a slight increase in PTH levels after incadronate treatment but a decrease after alfacalcidol treatment. The finding may suggest that incadronate is more appropriate than alfacalcidol as a drug for the treatment of osteoporosis. The results suggest that the pQCT method is sensitive enough to examine the effect of drugs on BMD without measuring the cortical bone BMD, which changes little during a 4-week study period (data not shown).

A 2-week measurement protocol was examined using deoxypyridinoline levels only. Ovariectomy significantly increased urinary deoxypyridinoline levels during the 2 weeks following surgery. Alfacalcidol had no significant inhibitory effect on urinary deoxypyridinoline levels, while incadronate dose dependently inhibited urinary deoxypyridinoline levels with significant inhibition occurring at a dose of 10 mg/kg (data not shown). This suggests that bone resorption is detectable as soon as 2 weeks after ovariectomy and that the effect of incadronate can be evaluated in such a short time. The effect of incadronate remained unchanged after the long-term treatment (data not shown).

Since there is a fundamental difference in the pharmacological mechanism between alfacalcidol (vit.D) and incadronate, further investigation would be needed to clarify the combined effect of both drugs.

Trabecular BMD measurement using the pQCT method is useful to estimate drug efficacy in a short period (4 weeks) as is the measurement of bone turnover makers.

Although alfacalcidol induced hypercalciuria and hypercalcemia, incadronate had no effect on urinary and blood calcium levels, suggesting that incadronate exerts its pharmacological effect (inhibition of bone resorption and increase in bone mass) by a mechanism different from that of alfacalcidol.

In conclusion, incadronate markedly inhibited the increase in bone resorption induced by ovariectomy, as measured either with bone turnover markers or BMD. The results also suggest that the drug acts directly to reduce bone resorption. Incadronate has an excellent safety profile, since

it does not induce hypercalcemia or hypercalciuria. Consequently, incadronate promises to be a safe and useful drug for the treatment of osteoporosis caused by stimulated bone resorption.

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