Prevention of Bone Loss by Bisphosphonate YM175 in Ovariectomized Dogs with Dietary Calcium Restriction

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ABSTRACT—We have evaluated the effects of YM175 {disodium dihydrogen (cycloheptylamino) methylenebisphosphonate monohydrate}, a novel bisphosphonate, on bone mineral densities (BMD) at the lumbar spine and forelimb in ovariectomized beagles with dietary calcium restriction. Groups 1 and 2 were given a sham operation and Groups 3–6 were ovariectomized. One month later (month 0), a low calcium diet was given to Groups 2–6. Groups 4–6 were orally treated with YM175 at doses of 0.01, 0.1 and 1.0 mg/kg, respectively, for 18 months. Changes in BMD at the lumbar spine and left forelimb were determined serially by dual energy X-ray absorptiometry. Calcium restriction decreased lumbar BMD by 19% at month 2 and by up to 30% at month 17 compared to its baseline value, but ovariectomy itself had a minimal effect on bone mass in dogs with restricted calcium intake. YM175 (1 mg/kg) prevented the bone loss at month 2 and YM175 at 0.1 mg/kg or more inhibited the BMD reduction at month 17. The magnitude of BMD reduction of the forelimb was less remarkable as compared to that of the lumbar spine. Urinary hydroxyproline excretion and plasma osteocalcin levels were increased by calcium restriction, indicating a high turnover of bone. YM175 reduced hydroxyproline excretion but not osteocalcin levels. These results indicate that YM175 prevents bone loss induced by calcium restriction and ovariectomy through partially normalizing high bone turnover.

Keywords: Bisphosphonate, YM175, Bone mineral density, Dietary calcium restriction, Ovariectomy

Cessation of ovarian function is a major cause of the development of postmenopausal osteoporosis (1, 2). Ovariectomized rats have been used extensively as a model for osteoporosis. In this animal model, the trabecular bone mass decreases significantly within several weeks (3–5). For this reason, it is appropriate to evaluate the efficacy of pharmacological agents on bone loss associated with estrogen deficiency in this model. However, the skeletal metabolism of rats is quite different from that of the adult humans because rat bone grows throughout their life period (6). Therefore, the information obtained from this model can not be directly extrapolated to the pathogenesis of human osteoporosis. It is necessary to develop a different animal model that exhibits human-like remodeling dynamics. The potential utility of ovariectomized dogs as a model of postmenopausal bone loss has been examined by several investigators (7–12) but the effects of ovariectomy on bone mass in dogs remain controversial. Some could detect significant bone loss (7–10), but others could not (11, 12). One possible explanation for the differing response of canine skeleton to estrogen deficiency would be attributed to the high calcium content in commercially available dog chow (13), as it has been reported that low dietary calcium increases the sensitivity to ovarian hormone deficiency in rats (14, 15) and minipigs (16). Thus, it is suggested that ovariectomized dogs with calcium restriction would be suitable for studying problems that are relevant to postmenopausal bone loss.

Bisphosphonates are analogues of pyrophosphates that contain P-C-P bonds, and they strongly bind to hydroxyapatite crystals and inhibit bone resorption (17, 18). The action of bisphosphonate has been clinically examined in different conditions characterized by increased bone resorption, such as humoral hypercalcemia of malignancy (19, 20), Paget's disease of bone (21) and osteoporosis (22, 23). YM175 {disodium dihydrogen (cycloheptylamino) methylenebisphosphonate monohydrate} is a

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novel bisphosphonate, and its efficacy has been elucidated in several different animal models of increased bone resorption: in animals immobilized by neurectomy, in experimental hypercalcemia induced by Walker-256 tumor and by parathyroid hormone administration (24–26). In our previous study, we have shown that YM175 preserved bone mass and strength in ovariectomized-calcium restricted beagle dogs (27). The purposes of the present study are to clarify the effects of dietary calcium restriction on serial changes in bone mineral densities in sham-operated or ovariectomized dogs and to evaluate the effects of the chronic treatment with YM175 on bone loss in ovariectomized dogs with dietary calcium restriction.

MATERIALS AND METHODS

Experimental animals

Forty of female beagle dogs at the age of 7 months were purchased from Hazleton LRE (Kalamazoo, MI, USA). During the 14 month-acclimatization period, they were individually housed and fed a standard dog chow containing 1.4% calcium, 0.9% phosphorus, and 200 IU/100 g of vitamin D (DS; Oriental Yeast, Tokyo). The mean of the body weight was increased from 8.6±0.1 to 11.2±0.2 kg during this period. Epiphyseal closure and adult skeletal status were confirmed by X-ray before the start of the experiment.

Experimental design

Twelve dogs at the age of 21 months were given a sham operation and divided into 2 groups (Groups 1–2). The remaining 28 dogs were ovariectomized (month −1) and divided into 4 groups (Groups 3–6). One month after the surgery (month 0), the diet for Groups 2–6 was changed to a low calcium diet (calcium: 0.14%; Oriental Yeast, Tokyo), which contained 1/10 the calcium contained in the standard diet. YM175 was packed into gelatin capsules and the volume was adjusted with lactose. The animals in Groups 4, 5 and 6 were orally given YM175 at doses of 0.01, 0.1 and 1.0 mg/kg, respectively, for 18 months (5 days a week), while Groups 1–3 received capsules containing lactose alone. Body weight was measured every two weeks. Tap water was given ad libitum and the amount of the food served was 300 g daily. The temperature in the room was maintained at 22±1℃. The experimental protocol was approved by the local animal ethics committee for animal studies.

Bone mineral density (BMD) measurement

BMD (g/cm^2) of the 2nd, 3rd and 4th lumbar vertebrae (L2–4) and left forelimb (radius and ulna) were measured by dual energy X-ray absorptiometry (DEXA) (XR-26; Norland Corporation, WI, USA) under xylazine-pentobarbital anesthesia. The averaged bone mass for L2–4 or for the whole radius and ulna (including epiphysis, metaphysis and diaphysis) were obtained before ovariectomy or sham operation and at 2, 5, 8, 11, 14 and 17 months following the start of calcium restriction. The final determination of BMD at the forelimb was conducted at month 18 instead of month 17. BMD of the lumbar spine or forelimb was also expressed as % of baseline BMD. The scan speed was set at 80 mm/sec. The point and line resolution was 1×1 mm for the lumbar spine or 1.5×1.5 mm for the forelimb, respectively. The coefficient of variation of five repeated scans conducted on the same preparation on the single day was 2.0% for the lumbar spine or 1.5% for the forelimb, respectively.

Plasma and urine chemistry

Blood samples were collected monthly with heparinized syringes. Plasma was separated by a brief centrifugation (1,800×*g* for 10 min) and stored at −80℃ until analysis. Plasma concentrations of calcium, inorganic phosphorus, alkaline phosphatase, urea nitrogen and total protein were determined by colorimetric methods using an auto-analyzer (model 736-10; Hitachi, Tokyo). The reagent kits used in this study were obtained from Daiichi Pure Chemicals (Tokyo). Plasma concentrations of 1,25-dihydroxyvitamin D {1,25-$(OH)_2$D} and osteocalcin were determined by radioreceptor assay and by radioimmunoassay (28), respectively (Teijin-Bio Laboratories, Tokyo). The amount of urinary hydroxyproline excreted for 24 hr was measured using the chloramine-T resin method (29). The hydroxyproline excretion was normalized for creatinine concentration (Creatinine test-Wako; Wako Pure Chemical Industries, Osaka).

Statistical analyses

Values are expressed as the mean±S.E.M. Analysis of data from Groups 1–3 and Groups 3–6 were performed separately by two-way repeated measures analysis of variance (ANOVA) in order to examine the time-dependent effects of calcium restriction, ovariectomy and YM175 on bone mass and the indices related to bone metabolism. If the interaction between the time and each treatment shown above was confirmed statistically, multiple comparisons between Groups 1–3 or Groups 3–6 were performed at the respective time intervals by Scheffe's test or Dunnet's multiple range test, respectively. A P value less than 0.05 was considered statistically significant.

RESULTS

General conditions

One dog in Group 4 died at the age of 31 months (month 10). The rest of the dogs (39) remained healthy

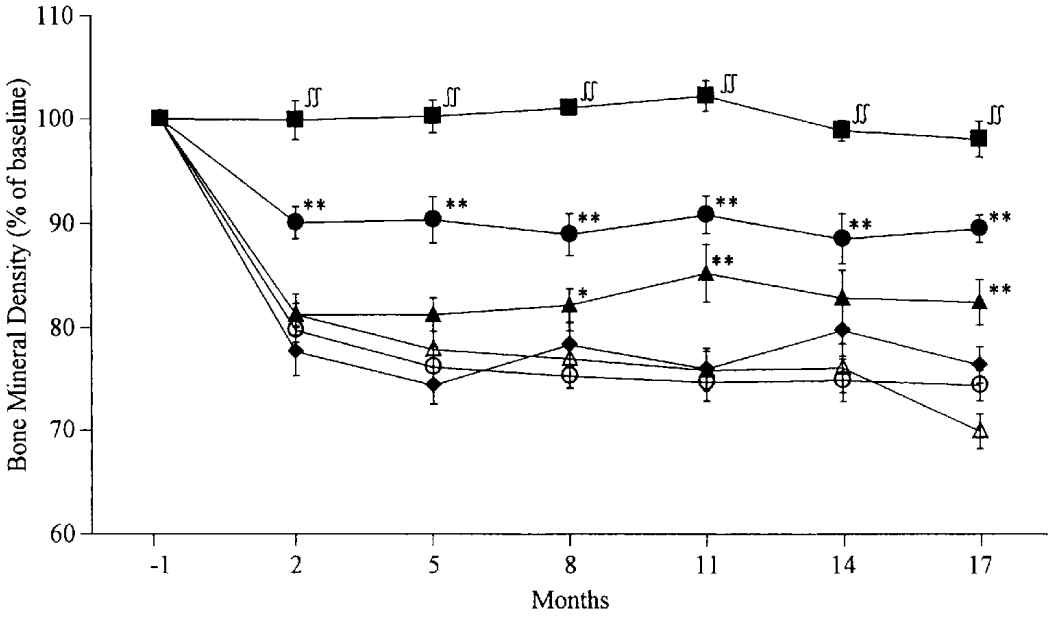

Fig. 1. Changes in bone mineral density of the lumbar spine (L2–4). The values are means±S.E.M. (n=6 or 7) which are expressed as % of baseline BMD. Group 1 (■): sham-operated and fed standard dog chow (calcium: 1.4%); Group 2 (△): sham-operated and fed a low calcium diet (calcium: 0.14%); Groups 3 (○), 4 (◆), 5 (▲), and 6 (●): ovariectomized and fed a low calcium diet; YM175 at doses of 0.01, 0.1 and 1.0 mg/kg was orally administered to Groups 4, 5 and 6 for 18 months. ʃʃ $P<0.01$ vs Group 3 (Scheffe's test). **$P<0.01$, *$P<0.05$ vs Group 3 (Dunnett's multiple range test).

until the end of the study. Consistent with the findings reported by others (7, 11), ovariectomized groups gained about 1.6–2.2 kg in body weight on the average during the study (data not shown). However, no weight gain was observed in Groups 1 and 2, showing that the standard or the low calcium diet had no effect on the body weight under our conditions.

Changes in BMD of lumbar spine

Figure 1 shows the mean changes in lumbar BMD for each group with time, which are expressed as a percent of the baseline BMD before ovariectomy or sham operation. The baseline value of lumbar BMD (month −1) in Group 1 was 0.584±0.031 g/cm^2, and this value was not significantly different from those of other groups (data not shown). BMD of Group 1 did not change significantly throughout the experimental period. Group 2 at month 2 exhibited a 19% reduction in lumbar BMD and it gradually decreased by up to 30% at month 17. Groups 2 and 3 exhibited comparable reductions in lumbar BMD, indicating that ovariectomy itself had a minimal effect in dogs fed the low calcium diet. Although there were no differences in lumbar BMD among Groups 3, 4 and 5 at month 2, BMD in Group 6 was significantly higher than that in Group 3, demonstrating that YM175 at a dose of 1.0 mg/kg partially but significantly prevented bone loss at month 2. In contrast to the slow continuous reduction in BMD in Group 3 after month 2, BMD in the groups treated with YM175 at doses of 0.1 and 1.0 mg/kg (Groups 5 and 6, respectively) were maintained at significantly higher levels than those in Group 3 at month 8 and thereafter. As shown in Fig. 2, YM175 dose-dependently prevented decreases in lumbar BMD at month 17, which were expressed as g/cm^2.

Changes in BMD of forelimb

The extent of forelimb BMD reduction in Group 3 was less remarkable as compared with those seen at the lumbar spine (Fig. 3). BMD in Group 3 was lower than that in Group 1 by 4.3% at month 2 and by 14.7% at month 18. In addition, Group 2 showed almost the same extent of BMD loss as those seen in Group 3 except in months 11 and 14. In contrast to the effects of YM175 on lumbar BMD reduction, YM175 ameliorated the reduction in BMD at month 18, whereas this agent had no effect on BMD in Group 6 at month 2 (Figs. 3 and 4).

Plasma and urine chemistry

It seems likely that 1,25-$(OH)_2$D levels in dogs fed a low calcium diet were increased according to an increase in the duration of the experiment (Fig. 5). The concentrations of 1,25-$(OH)_2$D in Groups 2 and 3 were consistently higher than those in Group 1. Neither ovariectomy nor YM175 had significant effect on 1,25-$(OH)_2$D levels. As shown in Table I, plasma alkaline phosphatase activity in Groups 2 and 3 were increased at month 2, but returned to

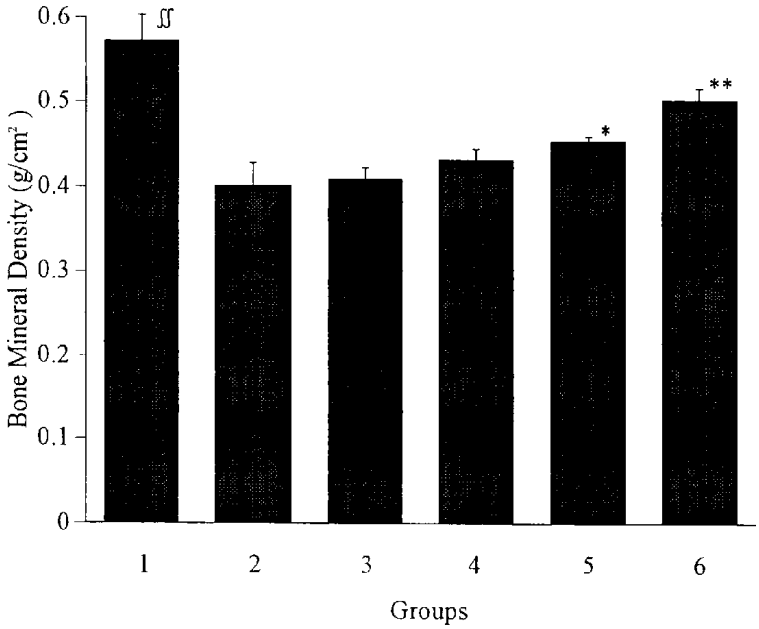

Fig. 2. Bone mineral density of the lumbar spine (L2–4) at month 17. Each bar with a vertical line shows the mean ± S.E.M. (n = 6 or 7) which is expressed as g/cm^2. Group 1: sham-operated and fed standard dog chow; Group 2: sham-operated and fed a low calcium diet; Groups 3–6: ovariectomized and fed a low calcium diet; the dose of YM175 (mg/kg): Group 3 (0), Group 4 (0.01), Group 5 (0.1), Group 6 (1.0). $^{\int\int}P < 0.01$ vs Group 3 (Scheffe's test). $^{**}P < 0.01$, $^{*}P < 0.05$ vs Group 3 (Dunnett's multiple range test).

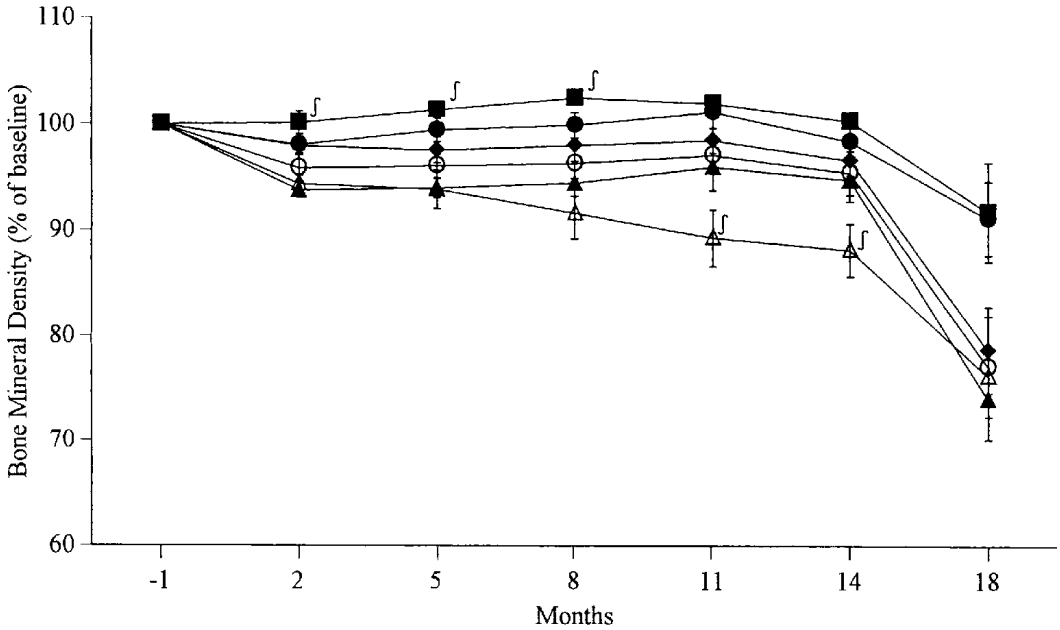

Fig. 3. Changes in bone mineral density of the forelimb (radius and ulna). The values are means ± S.E.M. (n = 6 or 7) which are expressed as % of baseline BMD. Group 1 (■): sham-operated and fed standard dog chow; Group 2 (△): sham-operated and fed a low calcium diet; Groups 3 (○), 4 (◆), 5 (▲) 6 (●): ovariectomized and fed a low calcium diet; the dose of YM175 (mg/kg): Group 3 (0), Group 4, (0.01), Group 5 (0.1), Group 6 (1.0). $^{\int}P < 0.05$ vs Group 3 (Scheffe's test).

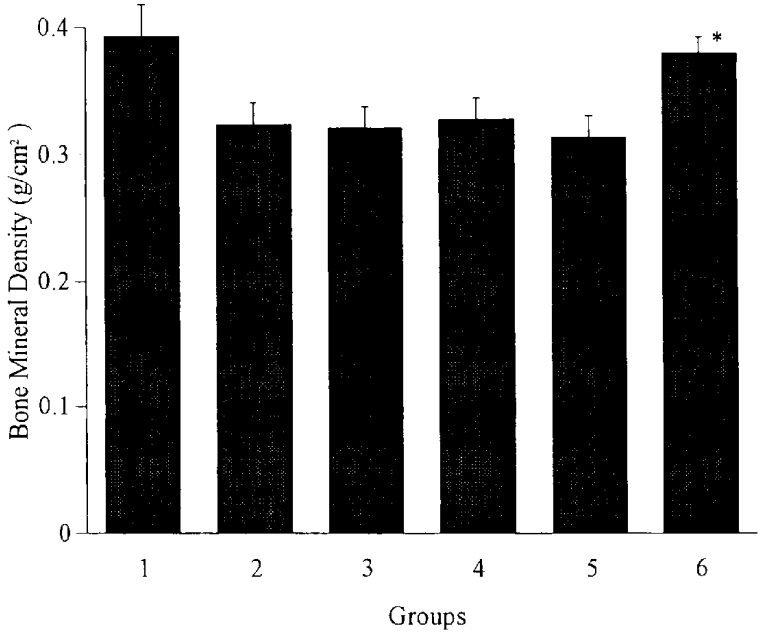

Fig. 4. Bone mineral density of the forelimb (radius and ulna) at month 18. Each bar with a vertical line shows the mean±S.E.M. (n=6 or 7) which is expressed as g/cm^2. Group 1: sham-operated and fed standard dog chow; Group 2: sham-operated and fed a low calcium diet; Groups 3–6: ovariectomized and fed a low calcium diet; the dose of YM175 (mg/kg): Group 3 (0), Group 4, (0.01), Group 5 (0.1), Group 6 (1.0). *$P<0.05$ vs Group 3 (Dunnett's multiple range test).

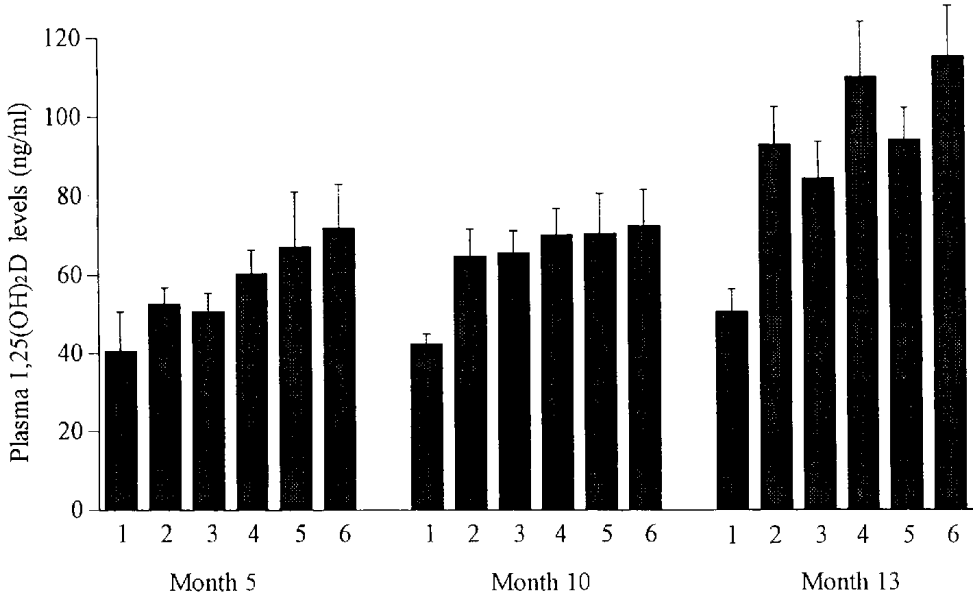

Fig. 5. Changes in plasma levels of 1,25-$(OH)_2$D. Each bar with a vertical line shows the mean±S.E.M. (n=6 or 7) Group 1: sham-operated and fed standard dog chow; Group 2: sham-operated and fed a low calcium diet; Groups 3–6: ovariectomized and fed a low calcium diet; the dose of YM175 (mg/kg): Group 3 (0), Group 4 (0.01), Group 5 (0.1), Group 6 (1.0).

the normal range at month 11. Other plasma parameters were not changed significantly throughout the study period. Urinary hydroxyproline and plasma osteocalcin levels are shown in Tables 2 and 3. Urinary hydroxyproline excretion tended to be increased in Groups 2–5 compared to Group 1 at month 2, but it decreased afterwards

Table 1. Plasma chemistry determined at months 2 and 11

Groups	Calcium (mEq/dl)		Phosphorus (mg/dl)		ALPase (KAU/dl)		Urea nitrogen (mg/dl)		Total protein (g/dl)	
	Month 2	Month 11	Month 2	Month 11	Month 2	Month 11	Month 2	Month 11	Month 2	Month 11
1	4.6±0.1	5.0±0.1	2.6±0.1	3.0±0.2	5.2±1.0	6.1±1.1	13.2±1.1	12.2±1.0	6.6±0.1	6.5±0.1
2	4.5±0.0	4.8±0.1	1.9±0.1	2.2±0.2	8.7±0.8[b]	7.2±0.8	11.5±1.2	10.3±0.9	6.5±0.2	6.7±0.2
3	4.5±0.0	4.9±0.1	2.3±0.2	2.9±0.8	8.9±0.6[b]	7.4±0.8	12.1±0.6	10.3±0.7	6.3±0.2	6.6±0.1
4	4.7±0.0	5.2±0.1	2.2±0.2	2.5±0.2	8.9±1.0	7.3±0.9	13.8±0.5	11.0±1.0	6.5±0.1	7.0±0.2
5	4.6±0.1	5.2±0.1	2.4±0.2	2.6±0.4	9.6±1.0	7.6±0.7	13.7±0.9	10.6±0.5	6.7±0.2	7.0±0.2
6	4.6±0.0	5.1±0.1	2.4±0.2	2.6±0.2	8.0±0.7	6.8±0.8	12.9±1.1	10.1±1.2	6.6±0.2	6.8±0.2

Each value is expressed as the mean±S.E.M. (n=6 or 7). ALPase: Alkaline phosphatase. [b]$P<0.05$ vs Group 1 (Scheffe's test).

Table 2. Urinary hydroxyproline excretion (μg/mg creatinine)

Groups	Baseline	Month 0	Month 2	Month 5	Month 8	Month 11	Month 14	Month 17
1	22.4±2.6	25.8±1.3	23.7±6.3	22.1±2.8	15.4±1.4	18.5±1.4	19.4±1.7	12.3±1.8
2	22.0±2.3	26.7±2.1	53.7±30.4	28.1±3.9	31.3±8.2	26.2±0.7	39.0±8.9	25.4±6.5
3	21.0±1.7	25.9±2.2	60.3±32.6	23.1±1.9	26.8±4.3	21.7±1.4	22.8±2.2	16.7±3.5
4	23.3±3.5	21.0±1.5	60.4±37.0	26.4±3.1	19.2±2.3	17.7±1.6	23.3±4.8	11.2±2.3
5	18.2±0.9	21.2±1.8	102.0±62.7	26.6±2.2	18.1±2.6	20.3±2.2	22.6±2.5	21.7±5.3
6	21.6±1.8	21.3±2.8	22.2±1.5	15.5±1.9	13.6±1.7	18.9±3.0	17.9±1.9	12.7±3.1

Each value is expressed as the mean±S.E.M. (n=6 or 7).

Table 3. Plasma osteocalcin levels (ng/ml)

Groups	Baseline	Month 2	Month 6	Month 10	Month 13	Month 18
1	24.0±2.0	15.5±1.5	10.5±0.5	12.1±0.5	11.9±0.6	17.6±2.0
2	18.8±1.5	29.3±1.8[a]	20.7±2.1[b,c]	17.6±1.5[a]	18.2±1.5[b]	29.7±4.0[b]
3	20.9±1.0	34.5±2.1[a]	29.7±3.0[a]	19.2±0.6[a]	21.0±2.0[a]	33.8±5.2[b]
4	18.5±2.0	38.3±1.6	31.4±2.5	22.0±2.2	22.6±1.5	27.3±3.1
5	21.0±2.0	35.4±2.6	28.6±2.3	19.7±1.2	19.5±1.3	28.5±2.9
6	22.3±1.6	35.1±2.0	22.3±1.8	17.1±1.2	19.0±1.3	29.9±2.9

Each value is expressed as the mean±S.E.M. (n=6 or 7). [a]$P<0.01$ vs Group 1, [b]$P<0.05$ vs Group 1, [c]$P<0.05$ vs Group 3 (Scheffe's test).

(Table 2). YM175 at 1.0 mg/kg inhibited the elevation of hydroxyproline levels throughout the experimental period. Plasma concentrations of osteocalcin in Groups 2 and 3 were significantly higher than that in Group 1, but no difference was observed between the two groups. YM175 had no significant effect on osteocalcin levels (Table 3).

DISCUSSION

The present study has demonstrated that dietary calcium restriction caused a marked bone loss at the lumbar spine in sham-operated or ovariectomized beagle dogs within 2 months after the start of the calcium restriction (Figs. 1 and 2). Lumbar BMD in sham-operated dogs (Group 2) was decreased by 19% at month 2 compared to baseline BMD, and an additional 11% of reduction was observed at month 17, leading to the total 30% reduction in lumbar BMD for 17 months. Ovariectomy itself may have a minimal effect on bone mass in dogs with restricted calcium intake because there was no statistically significant difference in lumbar BMD reduction between the two groups, without or with ovariectomy (Groups 2 and 3 in Fig. 1). The reduction in BMD of the forelimb was less remarkable as compared with those of the lumbar spine (Figs. 3 and 4). The BMD in Group 3 was lower than that in Group 1 by 4.3% at month 2 and by 14.7% at month 18. These data may suggest that the difference between the lumbar spine and forelimb regarding the time-course and the magnitude of bone loss was due to the different

composition of each bone. It is well-established that the turnover rate of trabecular bone is more rapid than that of cortical bone (5, 30). Since we measured BMD of whole lumbar spine and left forelimb in this study, the relative ratio of trabecular and cortical bones in each bone might be one of the most important determinants for bone turnover rate. Thus, our result showed that bone loss of the lumbar spine was more rapid than that of forelimb, suggesting that the turnover rate in the lumbar spine was higher than that in the forelimb.

Several studies have examined effects of ovariectomy on histomorphometric changes in cancellous and cortical bones in dogs. Although ovariectomy resulted in a significant bone loss in rats (3–5), the effects of ovariectomy on bone mass in dogs remain controversial (7–12). However, Nakamura and his associates (13, 31) reported the findings that ovariectomy resulted in the mechanical and histomorphological failure of the lumbar spine in dogs receiving a low calcium diet and then proposed the hypothesis that an excessive amount of calcium in the standard dog diets blunt the effect of ovariectomy in dogs. In our previous study (27), we have demonstrated that the sensitivity of bone to ovariectomy in dogs may not greatly increase 18 months after the restriction of calcium intake. Although the contribution of ovariectomy to the reduction in bone mass and mechanical strength at the organ level was small in this model, the histomorphometric examination identified that ovariectomy significantly increased trabecular separation, which was not increased by calcium restriction alone, indicating that ovariectomy deteriorated trabecular bone structure in dogs. In the present study, we have intensively examined the time-dependent effects of ovariectomy and calcium restriction on bone mass by measuring BMD at the lumbar spine and forelimb serially until 18 months. The results indicated that the reduction in lumbar BMD observed in this model was mainly caused by the calcium restriction. This is compatible with our previous bone mass and biomechanical study (27).

As shown in Figs. 1 and 2, YM175 at a dose of 1.0 mg/kg partially but significantly prevented lumbar BMD loss at month 2. YM175 at doses of 0.1 and 1.0 mg/kg prevented lumbar BMD loss at month 8 and thereafter. The magnitude of BMD reduction of the forelimb in Group 3 was less remarkable as compared with those seen at the lumbar spine (Fig. 3). YM175 did not ameliorate BMD reduction at the forelimb throughout the treatment period except in month 18 (Fig. 4). It is well-established that bisphosphonates are incorporated into bone rapidly and dose-dependently after the administration and that these agents showed a long half-life of skeletal retention (32). In addition, it is reported that bisphosphonates are distributed mainly on the resorption surface of bone where osteoclasts were observed (18). Therefore, the time-dependent and the tissue-specific effects of YM175 on bone resorption observed in this study could be due to the capability to accumulate sufficient YM175 to inhibit bone resorption in each bone. This is supported by the findings that the inhibitory effect of YM175 on bone resorption is directly proportional to the concentration of this agent in rat bone (25).

It is reported that there is an adaptive response in increasing 1,25-$(OH)_2$D production when the supply of dietary calcium was limited (33, 34). 1,25-$(OH)_2$D levels in dogs fed a low calcium diet were increased according to the duration of the experiment (Fig. 5). However, neither ovariectomy nor YM175 had significant effects on 1,25-$(OH)_2$D levels. The mechanism for the adaptive response in the production of 1,25-$(OH)_2$D is unknown. There was no significant change in plasma levels of calcium between all groups throughout the study (Table 1). However, plasma alkaline phosphatase activities in Groups 2 and 3 were increased at month 2, but gradually decreased at month 11. The low calcium diet caused a slight increase in urinary hydroxyproline excretion and also elevated plasma osteocalcin levels in these groups. These changes suggested that bone loss caused by a low calcium diet might to be attributable to the increased rate of bone turnover with resorption exceeding formation. YM175 reduced the increase in urinary hydroxyproline excretion but did not change elevated levels of plasma alkaline phosphatase and osteocalcin, suggesting that this agent suppressed bone resorption without affecting bone formation. This is compatible with our recent findings showing that the mineral apposition rate and bone formation rate were not greatly reduced by the treatment with YM175 as estimated by dynamic histomorphometry (27).

In conclusion, the present study showed that YM175 prevented bone loss in ovariectomized beagle dogs fed a low calcium diet and maintained bone mass as long as 17 months. Further investigation will be required to clarify the effect of YM175 on bone quality and the clinical significance of YM175 in preventing bone fracture in osteoporotic patients.

REFERENCES

1 Riggs BL and Melton LJ III: Involutional osteoporosis. N Engl J Med **314**, 1676–1686 (1986)
2 Dempster DW and Lindsay R: Pathogenesis of osteoporosis. Lancet **341**, 797–805 (1993)
3 Kalu DN: The ovariectomized rat model of postmenopausal bone loss. Bone Miner **15**, 175–192 (1991)
4 Wronski TJ, Dann LM, Scott KS and Cinton M: Long-term effect of ovariectomy and aging on the rat skeleton. Calcif Tissue Int **45**, 360–366 (1989)

5 Mosekilde L, Danielsen CC and Knudsen UB: The effect of aging and ovariectomy on the vertebral bone mass and biomechanical properties of mature rats. Bone **14**, 1–6 (1993)
6 Sontag W: Quantitative measurement of periosteal and cortical-endosteal bone formation and resorption in the midshaft of female rat femur. Bone 7, 55–62 (1986)
7 Martin RB, Butcher RL, Sherwood LL, Buckendahl P, Boyd RD, Farris D, Sharkey N and Dannucci G: Effects of ovariectomy in beagle dogs. Bone **8**, 23–31 (1987)
8 Malluche HH, Faugere M-C, Rush M and Friedler R: Osteoblastic insufficiency is responsible for maintenance of osteopenia after loss of ovarian function in experimental beagle dogs. Endocrinology **119**, 2649–2654 (1986)
9 Faugere M-C, Friedler RM, Fanti P and Malluche HH: Bone changes occurring early after cessation of ovarian function in beagle dogs: a histomorphometric study employing sequential biopsies. J Bone Miner Res **5**, 263–272 (1990)
10 Dannucci GA, Martin RB and Patterson-Buckendahl P: Ovariectomy and trabecular bone remodeling in the dog. Calcif Tissue Int **40**, 194–199 (1987)
11 Boyce RW, Franks AF, Jankowsky ML, Orcutt CM, Piacquadio AM, White JM and Bevan JA: Sequential histomorphometric changes in cancellous bone from ovariectomized dogs. J Bone Miner Res **5**, 947–953 (1990)
12 Shen V, Dempster DW, Birchman R, Mellish RWE, Church E, Kohn D and Lindsay R: Lack of changes in histomorphometric, bone mass, and biochemical parameters in ovario-hysterectomized dogs. Bone **13**, 311–316 (1992)
13 Nakamura T, Nagai Y, Yamato H, Suzuki K and Orimo H: Regulation of bone turnover and prevention of bone atrophy in ovariectomized beagle dogs by the administration of 24*R*,25(OH)$_2$D$_3$. Calcif Tissue Int **50**, 221–227 (1992)
14 Hodgkinson A, Aaron J, Horsman A, McLachlan MSF and Nordin BEC: Effect of oophorectomy and calcium deprivation on bone mass in the rat. Clin Sci Mol Med **54**, 439–446 (1978)
15 Kalu DN, Liu CC, Hardin RR and Hollis BW: The aged rat model of ovarian hormone deficiency bone loss. Endocrinology **124**, 7–16 (1989)
16 Mosekilde L, Weisbrode SE, Safron JA, Stills HF, Jankowsky ML, Ebert DC, Danielsen CC, Soggard CH, Franks AF, Stevens ML, Paddock CL and Boyce RW: Evaluation of the skeletal effect of combined mild dietary calcium restriction and ovariectomy in Sinclair S-1 minipigs. A pilot study. J Bone Miner Res **8**, 1311–1321 (1993)
17 Schenk R, Eggli P, Fleisch H and Rosini S: Quantitative morphometric evaluation of the inhibitory activity of new aminobisphosphonates on bone resorption in the rat. Calcif Tissue Int **38**, 342–349 (1986)
18 Sato M, Grasser W, Endo N, Akins R, Simmons H, Thompson DD, Golub E and Rodan GA: Bisphosphonate action. Alendronate localization in rat bone and effects on osteoclast ultrastructure. J Clin Invest **88**, 2095–2105 (1991)
19 Ralston SH, Patel U, Fraser WD, Gallacher SJ, Dryburgh FJ, Cowan RA and Boyle IT: Comparison of three intravenous bisphosphonates in cancer-associated hypercalcemia. Lancet **2**, 1180–1182 (1989)
20 Siris ES, Sherman WH, Baquiran DC, Schlatterer JP, Osserman EF and Camfield RE: Effect of dichloromethylene diphosphonate on skeletal mobilization of calcium in multiple myeloma. N Engl J Med **302**, 310–315 (1980)
21 O'Doherty DP, Bickerstaff DR, McCloskey EV, Hamdy NAT, Beneton MNC, Harris S, Mian M and Kanis JA: Treatment of Paget's disease of bone with aminohydroxybutylidene bisphosphonate. J Bone Miner Res **5**, 483–491 (1990)
22 Reginster JY, Deroisy R, Denis D, Collette J, Lecart MP, Sarlet N, Ethgen D and Franchimont P: Prevention of postmenopausal bone loss by tiludronate. Lancet **2**, 1469–1471 (1989)
23 Storm T, Thamsborg G, Steiniche T, Genant HK and Sorensen OH: Effect of intermittent cyclical etidronate therapy on bone mass and fracture rate in women with postmenopausal osteoporosis. N Engl J Med **322**, 1265–1271 (1990)
24 Nagao Y, Ishitobi Y, Fukushima S, Kinoshita H, Kawashima H and Kumegawa M: YM175 inhibits osteoclast differentiation and bone resorbing action of mature osteoclast. J Bone Miner Res **5**, Supp **2**, S159 (1990)
25 Kawamuki K, Abe T, Kudoh M, O'uchi N, Motoie H, Usui T, Isomura Y, Takeuchi M and Kawashima H: Effect of YM175 on bone positively correlates with its concentration in bone. J Bone Miner Res **5**, Supp **2**, S245 (1990)
26 Kudoh M, Abe T, Kawamuki K, Yamaoka E, Isomura Y, Takeuchi M and Kawashima H: Effect of YM175 on experimental hypercalcemia and tumor-induced osteolysis in rats. J Bone Miner Res **5**, Supp **2**, S166 (1990)
27 Motoie H, Nakamura T, O'uchi N, Nishikawa H, Kanoh H, Abe T and Kawashima H: Effects of the bisphosphonate YM175 on bone mineral density, strength, structure, and turnover in ovariectomized beagles on concomitant dietary calcium restriction. J Bone Miner Res **10**, 910–920 (1995)
28 Patterson-Allen P, Brautigam CE, Grindeland RE, Asling CW and Callahan PX: A specific radioimmunoassay for osteocalcin with advantageous species cross-reactivity. Anal Biochem **120**, 1–7 (1982)
29 Goverde BC and Veenkamp FJN: Routine assay of total hydroxyproline based on resin-catalysed hydrolysis. Clin Chim Acta **41**, 29–40 (1972)
30 Parfitt AM: The physiologic and clinical significance of bone histomorphometric data. *In* Bone Histomorphometry. Techniques and Interpretations, Edited by Recker RR, pp 143–223, CRC Press, Inc, Boca Raton (1983)
31 Yamaura M, Nakamura T, Nagai Y, Yoshihara A and Suzuki K: Reduced mechanical competence of bone by ovariectomy and its prevention with 24*R*,25-dihydroxyvitamin D$_3$ administration in beagles. Calcif Tissue Int **52**, 49–56 (1993)
32 Fleisch H: Bisphosphonates; Pharmacology and use in the treatment of tumour-induced hypercalcaemic and metastatic bone disease. Drugs **42**, 919–944 (1991)
33 Trechsel U, Eisman JA, Fischer JA, Bonjour J-P and Fleisch H: Calcium-dependent, parathyroid hormone-independent regulation of 1,25-dihydroxyvitamin D. Am J Physiol **239**, E119–E124 (1980)
34 Jaeger P, Jones W, Clemens TL and Hayslett JP: Evidence that calcitonin stimulates 1,25-dihydroxyvitamin D production and intestinal absorption of calcium in vivo. J Clin Invest **78**, 456–461 (1986)