

Morphometric Evidence That YM175, a Bisphosphonate, Reduces Trabecular Bone Resorption in Ovariectomized Dogs With Dietary Calcium Restriction

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ABSTRACT—We examined mechanisms by which incadronate disodium (YM175) prevented bone resorption in ovariectomized dogs with dietary calcium restriction using the morphometrical method. YM175 (0.01–1.0 mg/kg) was given to ovariectomized dogs for 18 months. Because lumbar bone mineral density remained constant at month 17, we assumed that the trabecular bone resorption rate was equal to the bone formation rate and that wall thickness equaled resorption cavity depth. YM175 decreased both the bone resorption rate per number of osteoclasts and resorption cavity depth of cancellous pockets which were increased in ovariectomized dogs. These results suggest that YM175 reduces bone loss by decreasing the resorbing activity of osteoclasts.

Keywords: Histomorphometry, Bone resorption, YM175

The cessation of ovarian function is a major cause of the development of postmenopausal osteoporosis (1). There is an increased risk of bone fracture associated with a reduction in bone mass and strength in women with osteoporosis. The stability of trabecular bone depends not only on bone mineral density (BMD), but also on the three-dimensional microstructure of the bone (2). Disruption of the microstructure of trabecular bone is one of the contributory factors to the pathogenesis of vertebra compression fractures (3). The potential utility of ovariectomized dogs as a model of postmenopausal bone loss is inconsistent. However, since the combination of ovariectomy and calcium deficiency causes more bone loss than ovariectomy in rats (4), it has been suggested that ovariectomized dogs with calcium restriction would be a suitable animal model for studying bone resorption.

YM175, disodium dihydrogen (cycloheptylamino) methylene bisphosphonate monohydrate (Yamanouchi, Tokyo) is a novel bisphosphonate, and its efficacy has been elucidated in several animal models of increased bone resorption (5, 6). Our previous studies demonstrated that the administration of YM175 for 18 months to ovariectomized dogs with dietary calcium restriction

reduced bone loss by partially normalizing the high bone turnover (7, 8). To elucidate the mechanism underlying the inhibition of bone resorption by YM175, a histomorphometrical analysis was carried out using the same canine model. Parameters concerning the resorption surface and resorption cavity depth in lumbar vertebrae were examined.

Forty female beagle dogs (7-month-old) were purchased from Hazleton LRE (Kalamazoo, MI, USA). The animals were fed a standard dog chow containing 1.4% calcium, 0.9% phosphorus, and 200 IU/100 g vitamin D₃ (DS; Oriental Yeast, Tokyo). Tap water was given ad libitum and the amount of the food served was 300 g daily. The dogs' body weights increased from 8.6 ± 0.1 to 11.2 ± 0.2 kg during the 14 month-acclimatization period. Epiphyseal closure and adult skeletal status were confirmed by X-ray before the start of the experiment. Twelve dogs at the age of 21 months were sham-operated and divided into Groups 1 and 2 (6 dogs each). The remaining 28 dogs were ovariectomized (month –1) and divided into 4 groups of 7 dogs each (Groups 3–6). Starting at one month after the surgery (month 0), the dogs in Groups 2–6 were given a low-calcium diet, which contained 1/10 the calcium in the standard diet. The animals in Groups 4–6 received orally YM175 in capsules

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at a dose of 0.01, 0.1 or 1 mg/kg from month 0 to month 17 (for 18 months, 5 days a week), during which time Groups 1–3 received lactose in capsules. The experimental protocol was approved by Yamanouchi ethics committee for the care and use of laboratory animals. At the end of the treatment, the dogs were anesthetized and killed by exsanguination.

All dogs were injected with tetracycline (20 mg/kg body wt., i.v.) 18 and 7 days before sacrifice. Specimens of L4 vertebral body were isolated by removing the posterior elements and transverse processes. The 4th lumbar vertebrae (L4) were fixed in 70% ethanol and embedded in methyl methacrylate after Villanueva's bone staining and then 8- μ m-thick sections of the specimen were made with a Polycut S microtome (Reichert Jung, Heidelberg, Germany). Morphometric indices of bone remodeling in trabecular bone were measured using an image analyzing system (MGA-4300; System-Supply, Nagano). In this study, we assumed that the bone resorption rate (BRR) was equal to the bone formation rate (BFR) because the BMD of the lumbar spine in Groups 2–6 remained unchanged at month 17 (8), and the cortical bone mass of the tibia was not significantly different among the groups (data not shown). The BRR is expressed per unit of bone volume (BRR/BV, %/year) or expressed per number of osteoclasts (BRR/N.Oc, %/year) (9). The BFR was obtained by multiplying the mineral apposition rate (MAR, μ m/day) by the mineralizing surface (MS/BS, %), which was calculated as the sum of the double-labeled surface and half of the single-labeled surface. The MAR was obtained by dividing the mean distance between the first and the second label by the interval of the labeling (11 days). The eroded surface (ES/BS) represented the percentage of the bone surface where osteoclasts were positive or negative. The wall thickness (W.Th) of cancellous packets was measured under polarized light. Only completed packets were measured at 4 points, which were at equal

intervals. The resorption period (Rs.P, days) was calculated according to the equation, $W.Th/MAR \times ES/BS \div \text{osteoid surface (OS/BS, \%)}$. The total period (Tt.P, days) was the sum of the remodeling period and the quiescent period and was calculated as $W.Th/MAR \div \text{mineralizing surface (MS/BS, \%)}$. The activation frequency (Ac.f, /year) was the reciprocal of Tt.P and indicated the probability that a new cycle of remodeling would be initiated at any point on the surface by the event of activation. The histomorphometric data (MAR and W.Th) were reported in three-dimensional terms with the correction factor of $\pi/4$ for the obliquity of the section.

For the measurement of the parameters related to osteoclasts, L5 vertebral bodies (L5) were fixed in 10% paraformaldehyde and decalcified with 10% EDTA for 30 days, followed by embedding in paraffin, sectioning 5- μ m-thick with a 2050 Supercut (Reichert-Jung) and staining with hematoxylin and eosin. The osteoclast surface (Oc.S/BS, %) is the percentage of the bone surface that consists of active eroded surfaces. The osteoclastic number (N.Oc/BS, mm^{-1}) is the number of osteoclasts per unit of bone surface. The BRR per osteoclast was calculated by dividing the BRR of L4 by the number of osteoclasts on L5, which was corrected by the ratio of the tissue volume of L4 to that of L5. All of these parameters were measured in a blind manner. The analyses of the data from Groups 1–3 and Groups 3–6 were performed separately by Student's *t*-test (Group 1 vs Groups 2–3) and one-way analysis of variance followed by Dunnett's multiple range test (Group 3 vs Groups 4–6).

Using morphometrical methods, we examined effects of YM175 on bone resorption in ovariectomized dogs with dietary calcium restriction. As shown in Table 1, the Oc.S/BS, N.Oc/BS and BRR/BV in Groups 2–3 were significantly increased compared to Group 1, although the BRR/N.Oc remained unchanged. These changes indicated that the bone resorption rate increased by calcium

Table 1. Effects of ovariectomy, calcium restriction and YM175 on bone resorption in canine vertebral bodies

Group	Treatment			ES/BS (%)	Oc.S/BS (%)	N.Oc/BS (mm^{-1})	BRR/BV (%/year)	BRR/N.Oc (%/year)
	OVX	Ca	YM175 (mg/kg)					
1	—	High	0	17.4 \pm 4.0	0.128 \pm 0.021	0.021 \pm 0.003	89.9 \pm 16.6	0.870 \pm 0.205
2	—	Low	0	16.8 \pm 0.8	0.264 \pm 0.093	0.053 \pm 0.008 ⁺⁺	327.4 \pm 14.6 ⁺⁺	1.110 \pm 0.241
3	+	Low	0	15.5 \pm 1.4	0.396 \pm 0.072 ⁺	0.080 \pm 0.024 ⁺	334.1 \pm 13.3 ⁺⁺	0.844 \pm 0.141
4	+	Low	0.01	14.8 \pm 1.4	0.471 \pm 0.072	0.077 \pm 0.014	272.1 \pm 20.5	0.611 \pm 0.074
5	+	Low	0.1	24.2 \pm 1.8*	0.552 \pm 0.033	0.084 \pm 0.008	239.5 \pm 35.0*	0.482 \pm 0.041*
6	+	Low	1.0	28.9 \pm 3.9**	0.961 \pm 0.317	0.153 \pm 0.046	131.5 \pm 28.4**	0.174 \pm 0.051**

OVX: Ovariectomy, Ca: Calcium content. Each value is the mean \pm S.E.M. (n=6 or 7). Groups 1 and 2: sham-operated and fed standard chow or a low-calcium diet; Groups 3–6: ovariectomized, fed a low-calcium diet and treated for 18 months with YM175 at doses of 0, 0.01, 0.1 and 1.0 mg/kg, respectively. ⁺⁺P<0.01, ⁺P<0.05 vs Group 1; ^{**}P<0.01, ^{*}P<0.05 vs Group 3.

restriction and/or ovariectomy might be due to increases in osteoclastic number. YM175 at a dose of 0.1 mg/kg or more significantly increased the ES/BS and tended to increase the Oc.S/BS and N.Oc/BS, but decreased BRR/BV and BRR/N.Oc dose-dependently. These findings indicated that although YM175 increased the eroded surface, it decreased the bone resorption per bone volume or per number of osteoclasts. The W.Th was significantly increased, the Rs.P and Tt.P were decreased, and the Ac.f was increased in Group 3, demonstrating that the activation interval and frequency of bone remodeling were increased (Table 2). In contrast, YM175 inhibited the increased resorption depth and normalized these indices, suggesting that YM175 decreased the depth of osteoclastic resorption cavities and normalized the bone turnover.

Our previous study (7) demonstrated that YM175 reduced trabecular bone loss in L2–4 vertebral bodies of ovariectomized dogs with dietary calcium restriction. This agent also increased the trabecular bone volume and trabecular bone thickness that were decreased in this model. The precise mechanism by which YM175 reduced the bone loss remains unclear, but this agent normalized the increased bone turnover caused by ovariectomy and calcium restriction. The morphometric analysis showed that YM175 reduced the bone loss by decreasing BRR/N.Oc and the depth of osteoclastic resorption cavities. Several reports, however, showed that bisphosphonates decreased the osteoclast number (10). On the other hand, YM175 increased Oc.S/BS and N.Oc/BS in this canine model. Similar findings with other bisphosphonates were reported (11). Although there is no definite explanation for this difference among several bisphosphonates about changes in ES/BS and N.Oc/BS, the eroded surface increased by YM175 may be due, at least in part, to the

increased number of osteoclasts in compensation for their attenuated ability of bone resorption.

Bone resorption is the product of resorption surface by resorption depth. YM175 treatment increased ES/BS and reduced BRR/BV, suggesting that the main effect of YM175 was the reduction of the bone resorption depth. As mentioned above, we assumed that W.Th equaled bone resorption depth in this study. In accord with the findings by Devlin et al. (12), the bone resorption depth was increased following ovariectomy and calcium restriction. Such an increase in W.Th was reversed by YM175 dose-dependently. YM175 decreased the bone turnover, which was estimated by BRR/BV and BFR/BV. Contrary to our findings, other bisphosphonates such as alendronate and etidronate, increased W.Th after long-term treatment (13) or had no effect on W.Th (14). A significant increase in wall thickness was detected in transiliac biopsy specimens obtained from postmenopausal women treated with alendronate for 24 months (15). Etidronate, in contrast, had no effect on W.Th in postmenopausal osteoporosis patients after 60 weeks of treatment (14). Further study is necessary to examine the effect of YM175 on bone balance in the clinical setting.

In conclusion, YM175 decreased trabecular bone loss by inhibiting the function of osteoclasts, which increased bone resorption rate and the depth of resorption cavities.

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Table 2. Effects of ovariectomy, calcium restriction and YM175 on wall thickness and bone turnover in canine vertebral bodies

Groups	W.Th (μ m)	Rs.P (days)	Tt.P (days)	Ac.f (/year)
1	13.7 \pm 0.9	21.7 \pm 5.5	131.1 \pm 27.1	3.29 \pm 0.53
2	16.2 \pm 1.0	7.3 \pm 0.5 ⁺	43.7 \pm 3.0 ⁺	8.55 \pm 0.60 ⁺⁺
3	16.7 \pm 0.5 ⁺	6.8 \pm 0.8 ⁺	43.3 \pm 2.0 ⁺	8.53 \pm 0.38 ⁺⁺
4	15.3 \pm 0.5	6.7 \pm 0.3	47.2 \pm 4.5	8.06 \pm 0.70
5	14.5 \pm 0.9*	11.8 \pm 0.7	49.3 \pm 2.7	7.52 \pm 0.37
6	10.6 \pm 0.3**	23.2 \pm 6.4**	83.0 \pm 21.9	6.41 \pm 1.61

Each value is the mean \pm S.E.M. (n=6 or 7). Groups 1 and 2: sham-operated and fed standard chow or a low-calcium diet; Groups 3–6: ovariectomized, fed a low-calcium diet and treated for 18 months with YM175 at doses of 0, 0.01, 0.1 and 1.0 mg/kg, respectively. ⁺⁺P<0.01, ⁺P<0.05 vs Group 1; ^{**}P<0.01, ^{*}P<0.05 vs Group 3.

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