Curative Effect of Combined Treatment With Alendronate and 1α-Hydroxyvitamin D₃ on Bone Loss by Ovariectomy in Aged Rats

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ABSTRACT—We investigated the combined effects of alendronate and 1α-hydroxyvitamin D₃ (1α(OH)D₃) on the bone mass and strength in aged ovariectomized rats and compared them with those of single treatments. Forty-week-old female rats underwent ovariectomy or sham operation, and after 15 weeks, ovariectomized rats were daily administered vehicle alone, alendronate (0.2 or 1.0 mg/kg, p.o.), 1α(OH)D₃ (0.02 μg/kg, p.o.), or the combinations of 0.2 or 1.0 mg/kg of alendronate and 1α(OH)D₃. After 12 weeks, the groups receiving combined treatments had significantly increased bone density and mechanical strength of the 4th lumbar vertebral body and the midfemur compared to the vehicle-treated group, and the effects were almost equal to or slightly less than the addition of those of the respective single treatments. The increase in mechanical strength was proportional to that in bone mineral density, suggesting that the stimulatory effects of these treatments on bone strength are ascribable primarily to those on bone mass. Analyses of histology, computed tomography, and biochemical markers confirmed the strong effect of the combined treatment on trabecular bone in particular, which was associated with increased trabecular number and decreased bone turnover. We propose that the combination of daily alendronate and 1α(OH)D₃ is clinically promising as a curative treatment of established postmenopausal osteoporosis.

Keywords: Combined treatment, Alendronate, 1α-Hydroxyvitamin D₃, Ovariectomy, Osteoporosis

Estrogen deficiency after menopause is associated with bone loss leading to fractures that constitute a serious health problem in many countries (1). Many bone-sparing drugs have successively been developed for the treatment of postmenopausal osteoporosis (2). However, the effects of the combined treatments of these drugs have not been well investigated despite the fact that postmenopausal osteoporosis is frequently treated with combination therapy.

Bisphosphonates, potent anti-resorptive agents, are known to be one of the most reliable and prevalent drugs in osteoporosis treatment. Alendronate (sodium 4-amino-1-hydroxybutilidene-1,1-bisphosphonate trihydrate), an aminobisphosphonate, is well known as a potent inhibitor of osteoclastic bone resorption (3 – 5). The efficacy of bisphosphonates has been demonstrated in osteoporosis (6 – 8) as well as in various other metabolic bone disorders such as Paget's disease (9) and hypercalcemia of malig-

nancy (10). Alendronate has also been reported to increase the bone mass and strength in ovariectomized (OVX) rats (11 – 13). The modes of action of alendronate include suppression of the differentiation of osteoclast precursors (14 – 16) and the activation of mature osteoclast function (17), resulting in the decrease in bone turnover. Recent studies on its molecular mechanism show that aminobisphosphonates such as alendronate can inhibit enzymes of the mevalonate pathway (18).

Vitamin D and vitamin D derivatives may also have a role in osteoporosis (19), and the activated form of vitamin D (1,25(OH)₂D₃ or calcitriol) can significantly improve bone mass and reduce vertebral fracture rates in postmenopausal women (20 – 22). 1α-Hydroxyvitamin D₃ (1α(OH)D₃ or alfacalcidol) is a prodrug of 1,25(OH)₂D₃ that is metabolized into 1,25(OH)₂D₃ in the liver and in osteoblastic cells (23, 24). This agent exhibits a bone sparing effect similar to that of 1,25(OH)₂D₃, and it is used for the treatment of postmenopausal osteoporosis primarily in Japan and European countries (25 – 29). 1α(OH)D₃ has

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been reported to prevent bone loss and increase the mechanical properties in ovariectomized rats, although its mechanism is still controversial (30 – 32). $1\alpha(OH)D_3$ as well as $1,25(OH)_2D_3$ increases bone mass possibly by up-regulating the intestinal absorption of calcium or modulating bone turnover (33).

We recently reported that the combined treatment with etidronate, a rudimentary bisphosphonate, and $1\alpha(OH)D_3$ increased bone mass and strength additively in OVX rats (34). However, the effect shown in the previous study was not curative but preventive for bone loss by OVX, because the application of etidronate and/or $1\alpha(OH)D_3$ started only one week after the operation. In addition, the rats used in the study were 14-week-old when bone growth and maturation had not yet been completed (35, 36). To investigate the curative effect of the combined treatment with bisphosphonates and $1\alpha(OH)D_3$ on established postmenopausal osteoporosis, in the present study we ovariectomized 40-week-old rats and the application of agents started 15 weeks after the surgery. As a bisphosphonate, this time we used alendronate that inhibits bone resorption more potently (11, 37), and is now used more prevalently for osteoporosis than etidronate. We evaluated the effects of the combined treatment with alendronate and $1\alpha(OH)D_3$ on bone mass and strength in these aged OVX rats and compared them with those of single treatments with each drug.

MATERIALS AND METHODS

Materials

Alendronate and $1\alpha(OH)D_3$ were synthesized by Merck Research Laboratories (West Point, PA, USA) and Teijin, Ltd. (Tokyo), respectively. All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) unless otherwise stated. Alendronate was dissolved in phosphate-buffered saline. The $1\alpha(OH)D_3$ solution was first diluted with 0.1% Triton X-100 in saline solution, and this solution was further dissolved with ethanol to the concentration of 5% (vol/vol). Equal volume of each solution of alendronate and $1\alpha(OH)D_3$ or the respective vehicle was mixed just prior to administration. The final ethanol concentration of administration solution was adjusted to 2.5%.

Experimental design

All animal experiments were performed according to the guidelines of the International Association for the Study of Pain (38). In addition, the experimental work was reviewed by the committee of Teijin Institute for Bio-medical Research charged with confirming ethics. Female Sprague-Dawley rats were purchased from Charles River Japan, Ltd. (Yokohama), and they were fed standard laboratory chow (CE-2; Clea Co., Tokyo) containing 1.18 g calcium, 1.03 g phosphorus and 250 IU vitamin D_3 added per 100 g dry weight. They were acclimated in an animal room maintained at a temperature of $24 \pm 2°C$ and a humidity of $55 \pm 10\%$ with a 12-h light / 12-h dark cycle, and were given tap water in bottles ad libitum. The rats were divided into two groups at 40 weeks of age: one group was ovariectomized (n = 53) and the other sham-operated (n = 13). Fourteen weeks later (54 weeks of age), the rats were subjected to dual energy X-ray absorptiometry (DEXA) to confirm the bone loss of the lumbar spine (L2 – L5) as described below. The L2 – L5 bone mineral density (BMD) of OVX rats was significantly decreased as compared to that of sham-operated rats (0.212 ± 0.016 vs 0.246 ± 0.020 g/cm^2, mean $\pm$ S.D., respectively, $P<0.001$ by Student's two-tailed t-test). The OVX rats were assigned to 6 groups in such a way that there were no significant differences in the L2 – L5 BMD and the body weight among groups. One week thereafter (55 weeks of age), the 6 groups of OVX rats were administered daily oral doses of the following: vehicle alone (OVX, n = 9), 0.2 mg/kg of alendronate (ALN-L, n = 8), 1.0 mg/kg of alendronate (ALN-H, n = 9), 0.02 μg/kg of $1\alpha(OH)D_3$ (1α, n = 9), combination of ALN-L and 1α (ALN-L + 1α, n = 9), or that of ALN-H and 1α (ALN-H + 1α, n = 9). The sham-operated rats received vehicle alone (Sham, n = 13). After 12 weeks of administration, all rats were euthanized for the following analysis. The body weight of each animal was measured once a week until the final day of administration.

Plasma and urine biochemistry

On the final day of administration, the rats were anesthetized under ether, and blood was collected from the abdominal aorta into a heparinized syringe. It was then centrifuged at 3000 rpm for 10 min at 20°C to separate the plasma for measurement of the calcium concentration with an autoanalyzer (Type 7070; Hitachi Co., Tokyo). Moreover, intact osteocalcin (I-OC) levels were measured as described before (39). Plasma I-OC is widely used as a specific marker of bone formation as it is synthesized predominantly by the osteoblasts, the bone-forming cells (39). Twenty-four-hour urine samples were collected from 6 randomly selected rats of each of the 7 groups. The animals were housed in metabolic cages for 2 days before the sacrifice for measurement of deoxypyridinoline (D-Pyr) by a high-performance liquid chromatography (HPLC) system as described earlier (40). Urinary D-Pyr is widely used as a specific marker of bone resorption as it is released from bone matrix during its degradation by the osteoclasts, the bone-resorbing cells. The urinary D-Pyr values were corrected for urinary creatinine (CRE) concentrations and were expressed as pmol/μmol CRE. Calcium and CRE concentrations were measured with the same autoanalyzer

used for plasma samples.

Bone mineral density (BMD)

BMD (g/cm²) was measured by DEXA (QDR-2000; Hologic, Waltham, MA, USA). For the adjustment of BMD of OVX rats among groups before starting treatment, L2 – L5 BMD in vivo was measured according to Ammann's methods (41) under sodium pentobarbital anesthesia. To assess effects of the treatments, ex vivo BMD was measured on excised L4 vertebral bodies and right femurs cleaned of soft tissues. After removing the adhering soft tissues, the length of the femur and height of the L4 vertebral body were measured with a micrometer. The L4 vertebral body was isolated by removing posterior elements, and the BMD was measured with antero-posterior application of radiation beams to the specimen. The region of interest of the femur was divided longitudinally into five equal fields (R1 – R5), and the BMD of R3 (midportion of femur) was measured. Scans were made in the regional high-resolution mode for small animal scanning with a 0.254-mm line spacing and a 0.127-mm point resolution as described (11). The coefficient of variation for the measurement of BMD of standard samples by this technique was 1.0%.

Mechanical testing

A load torsion tester (Model MZ-500D; Marutoh Co., Ltd., Tokyo) was employed for biomechanical tests. To analyze the mechanical strength of the L4 vertebral body, an axial compression test was performed according to a previously described method (42, 43). Briefly, all adhering soft tissue was carefully removed from the vertebra, and L4 was removed after separation from the adjacent discs with a scalpel. All vertebral processes were removed with a saw. The caudal end of the vertebral body was then embedded in polymethylmethacrylate (OSTRON-II; GC Dental Products Co., Aichi). The actuator (5 mm) was then displaced at a rate of 6 mm/min until fracture occurred as described (11). A three-point bending test was performed as described previously to analyze the mechanical strength of the midshaft of the femur (44). Briefly, all adhering soft tissues were carefully removed from the left femur and positioned so that one fulcrum was at the distal 1/5 part of the total length and the other was at the proximal 4/5 part of it. The bending force was applied with the cross head at the speed of 6 mm/min until fracture occurred. The ultimate load was determined directly from the load deformation curve and expressed in Newton (N) units.

Relationship between BMD and mechanical properties

We completed power-law regression analyses of the relationships of the ultimate load to BMD values of the L4 vertebral body and the midfemur as described before (34,

45). The relationship between the values of the ultimate load and BMD was analyzed by the equation: $S = C \times D^a$, where S is ultimate load, D is BMD, *a* is the experimentally derived value for power relationship, and C is the experimentally derived value of constant. Since this equation can also be written as $\log S = a \times \log D + \log C$, the regression analyses were performed between logS and logD. The values of the regression slope (*a*), correlation coefficient (*r*) and correlation significance (*P*) were obtained.

Microcomputed tomography

A microcomputed tomography scan of the femur and the L2 vertebral body specimens of a representative of each of the 7 groups was taken using equipment (Model NX-CP-C80H-IL) developed by Nittetsu ELEX Co., Ltd. (Osaka) as previously described (46). Femur specimens were evaluated at the midportion and at 10 mm from the distal end by cross-sectional analysis. The L2 vertebral body specimen was measured at the center by sagittal section.

Histological analyses

Fluorescence labeling was performed by subcutaneous injection with 25 mg/kg of tetracycline (Pfizer Pharmaceuticals Co., Tokyo) at 10 days and with 10 mg/kg of calcein (Dojin, Co., Tokyo) 2 days before sacrifice. Seven randomly selected rats/group were analyzed. The proximal end (1.2 – 1.5-cm-long) of the tibia was cut and its anterior eminence was removed prior to processing. Using an automated tissue processor, the tissues were dehydrated through graded alcohol and xylene and then infiltrated with and embedded in methylmethacrylate (MMA). A motorized microtome was used to cut sagittal sections of L2 and longitudinal frontal sections of the proximal tibia into 5 – 7-μm sections, which were then stained with von Kossa as described by Schenk et al. (47). Parameters were measured with an image analyzer (BIOQUANT Image Analysis Corp., Nashville, TN, USA) linked with a microscope equipped with bright field and epifluorescence optics and a polarization attachment. Measurements in the proximal tibia included an area situated between two lines traced parallel and approximately 1 and 4 mm distal to the growth plate. For the cancellous bone of the vertebra, a ±0.3-mm-thick line was traced at the inner edge of both epiphysial growth plates. All cancellous bone tissue between the traced lines was measured, with the exception of a ±0.3-mm-thick transitional zone lying between the cortical and cancellous bone tissue. The following parameters were measured as described before (48, 49). The static parameters: trabecular bone volume/tissue volume (BV/TV, %), trabecular thickness (Tb.Th, μm), and trabecular number (Tb.N, /mm). The dynamic parameters were as follows: mineral apposition rate (MAR, μm/day), mineralizing surface (MS/BS,

%), and bone formation rate (BFR/BS, $\mu m^3/\mu m^2$ per day) calculated by MAR × MS/BS. These parameters were calculated by measuring the tetracycline and/or calcein labeled surface and the average distance of two lines.

Statistical analyses

Data are expressed as the means ± S.D. The comparison between Sham and OVX groups was done by Student's two-tailed *t*-test. Comparisons among treated groups were statistically processed by the one-way analysis of variance (ANOVA) with Tukey's post hoc analysis in the SAS software package (SAS Institute Japan Ltd., Tokyo). *P* values less than 0.05 were considered significant. Correlation between BMD and ultimate load was assessed using regression analysis.

RESULTS

Body weight and biochemical markers

On the day prior to the start of treatment (15 weeks after OVX), the body weight of OVX rats was significantly greater than that in the Sham group (Table 1). This difference had become greater at the end of the experiment, and none of the single or combined treatments fully restored the balance. Both plasma and urinary calcium levels were lower in the OVX group compared to those in the Sham group. The single treatment with $1\alpha(OH)D_3$ increased the urinary calcium level. The plasma calcium levels of the combined treatment groups were significantly lower than that of the OVX group. Both plasma intact osteocalcin, a marker of bone formation, and urinary deoxypyridinoline, a marker of bone resorption, levels were increased by OVX compared to those in the Sham group, suggesting a high-turnover state. Levels of these markers were not affected by $1\alpha(OH)D_3$, but tended to be decreased by the single and combined treatments with alendronate. Urinary deoxypyri-

dinoline levels were significantly decreased by combined treatments as compared to respective single treatments.

BMD

There were no significant differences in the lengths of tibia and femur or in the heights of L2 and L4 vertebral bodies among the groups (data not shown). To examine the effects of single and combined treatments on bone mass, BMDs of the L4 vertebral body and the midportion of the femur were measured (Fig. 1). In the OVX group, these were 24.2% and 9.4% lower at L4 and midfemur, respectively, than BMD in the Sham group (both $P<0.05$), suggesting that estrogen deficiency for 27 weeks decreased both trabecular and cortical bones. Treatment with alendronate alone failed to affect BMDs and $1\alpha(OH)D_3$ increased BMD at the midfemur only, compared to OVX. No significant differences were observed among these three single treatment groups. The combined treatments, however, significantly increased BMD compared to OVX, and the effects were almost equal to or slightly less than the addition of those of respective single treatments. The ALN-L + 1α group showed significantly higher BMD of midfemur than that of the ALN-L group. These results indicate that the combined treatments are beneficial for the mass of both trabecular and cortical bones that were decreased by OVX.

Mechanical strength

The ultimate loads of both the L4 vertebral body and the midfemur were significantly decreased by OVX as compared to the Sham group (Fig. 2). Among single treatment groups only the ALN-H group showed significantly higher mechanical strength than the OVX group in the L4 vertebral body. Combined treatments also increased mechanical strength significantly in both bones compared to OVX. In the L4 vertebral body, the effects were almost equal to the addition of those of respective single treatment groups, and

Table 1. Effects on body weight and biochemical markers

Group	Body weight Pre-treatment (g)	Post-treatment (g)	Plasma calcium (mg/dL)	Urinary calcium (mg)	I-OC (ng/mL)	D-Pyr (pM/μM CRE)
Sham	477.0 ± 83.3[a]	483.9 ± 57.3[a]	10.73 ± 0.38[a]	56.5 ± 28.1[a]	20.3 ± 3.0[a]	9.7 ± 2.1[a]
OVX	550.2 ± 38.8	612.2 ± 58.9	10.23 ± 0.31	18.4 ± 7.5	32.5 ± 7.8	14.0 ± 2.6
ALN-L	480.7 ± 39.6	505.8 ± 36.4	10.08 ± 0.28	15.2 ± 8.5	26.1 ± 4.8	11.6 ± 2.4
ALN-H	506.8 ± 62.0	543.1 ± 76.6	10.16 ± 0.34	10.9 ± 4.6	23.5 ± 4.7[b]	9.9 ± 4.1
1α	535.1 ± 93.0	540.9 ± 116.9	10.72 ± 0.68[c]	60.0 ± 16.4[bcd]	27.0 ± 5.3	11.4 ± 1.6
ALN-L + 1α	512.6 ± 60.1	542.3 ± 72.9	9.73 ± 0.28[e]	64.7 ± 43.4[bcd]	27.7 ± 9.4	5.5 ± 1.1[bcde]
ALN-H + 1α	530.0 ± 77.0	557.4 ± 87.6	9.60 ± 0.33[bde]	52.1 ± 26.1[d]	21.7 ± 4.5[b]	5.7 ± 1.0[bcde]

I-OC, plasma intact osteocalcin; D-Pyr, urinary deoxypyridinoline; CRE, urinary creatine. Pre-treatment shows the data on the day prior to the start of treatment. Post-treatment shows those on the final day of the treatment. [a]$P<0.05$ vs OVX group (Student's two-tailed *t*-test). [b,c,d,e]$P<0.05$ vs OVX, ALN-L, ALN-H, 1α groups, respectively (Tukey's test). Data are expressed as the mean ± S.D. n = 8 – 13, except for Urinary calcium and D-Pyr (n = 6).
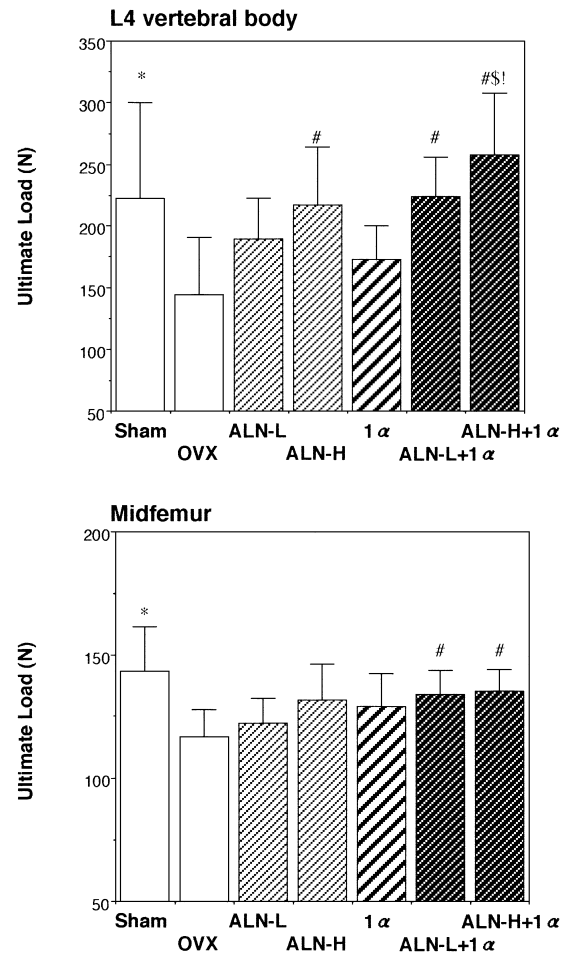
L4 vertebral body
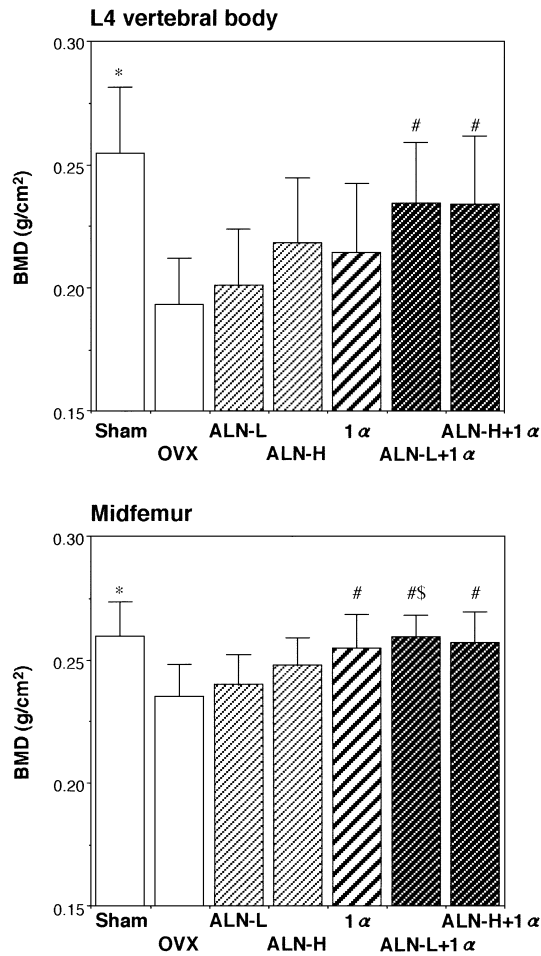
Midfemur

Fig. 1. Bone mineral density (BMD) of the L4 vertebral body and the midfemur. BMD of excised L4 vertebral body and right midfemur was measured by DEXA after 12 weeks of the indicated treatments. Data are expressed as the mean (bars) ± S.D. (error bars) of 8 – 13 bones/group. *$P<0.05$ vs OVX group (Student's two-tailed t-test). $^{#,\$}P<0.05$ vs OVX, ALN-L groups, respectively (Tukey's test).
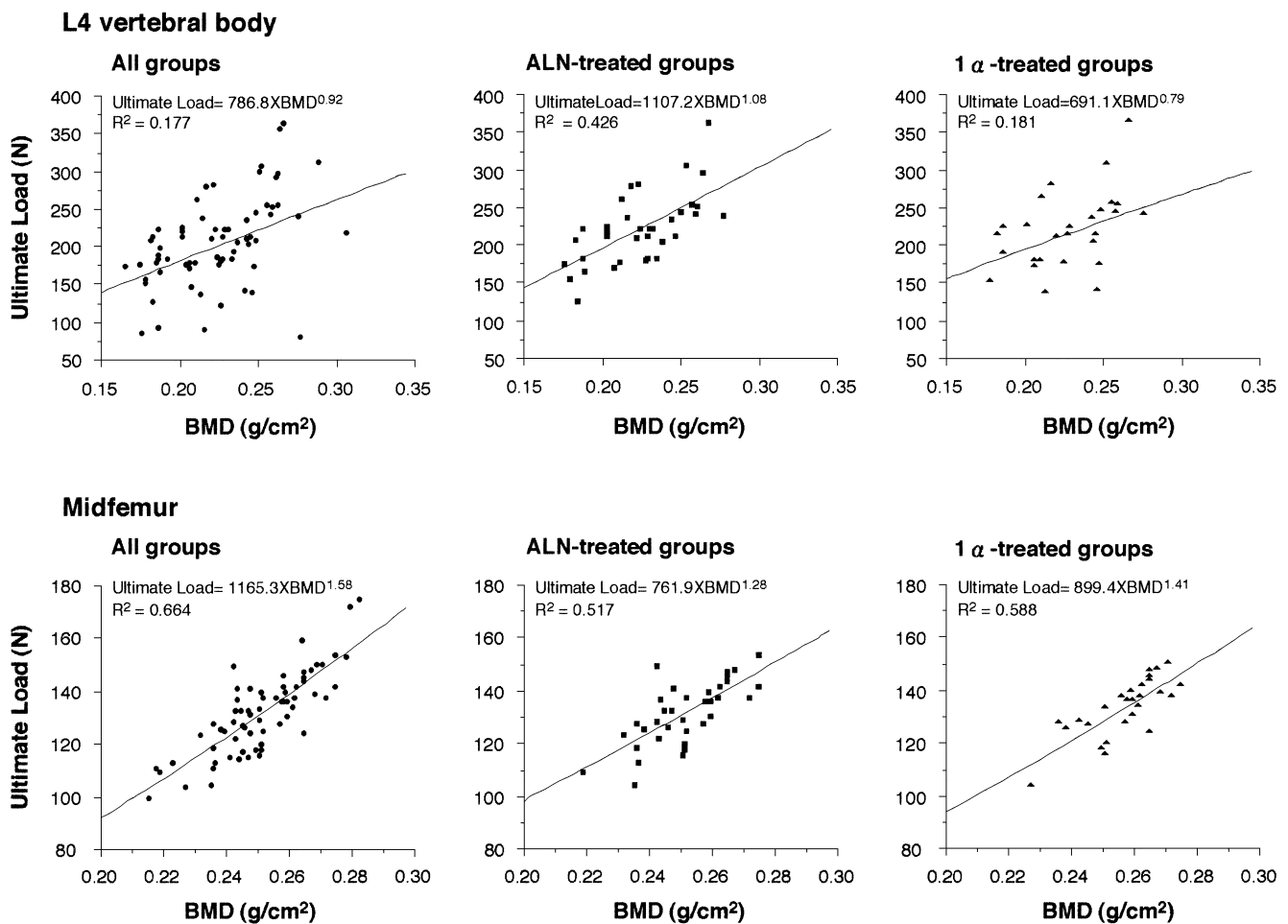
L4 vertebral body

Midfemur

Fig. 2. Mechanical strengths of the L4 vertebral body and the midfemur. Mechanical strength was determined by the ultimate load (N) after 12 weeks of the indicated treatments. An axial compression test and a three-point bending test were performed for the analyses of mechanical strengths of the L4 vertebral body and the midfemur, respectively, as described in Materials and Methods. Data are expressed as the mean (bars) ± S.D. (error bars) of 8 – 13 bones /group. *$P<0.05$ vs OVX group (Student's two-tailed t-test). $^{#,\$,!}P<0.05$ vs OVX, ALN-L, 1α groups, respectively (Tukey's test).

the ALN-H + 1α group showed significantly higher ultimate load than those of the ALN-L and 1α groups. In the midfemur, however, the effects of the combined treatments were similar to those of the single treatments, suggesting that the combined treatments are more effective on the mechanical strength of trabecular bone than that of the cortical bone.

Relationship between BMD and mechanical strength

To study the contribution of the effects of these treatments on bone mass to those on mechanical strength in OVX rats, power-law regression analyses of the relationship of BMD and the ultimate load of the L4 vertebral body and the midfemur were carried out. In addition to the analysis of all groups, separate analyses were performed on non-treated groups (Sham & OVX), alendronate-treated groups (ALN-L, ALN-H, ALN-L + 1α, ALN-H + 1α), and

$1\alpha(OH)D_3$-treated groups (1α, ALN-L + 1α, ALN-H + 1α). In all groups, Sham-OVX, the alendronate-treated groups, and $1\alpha(OH)D_3$-treated groups, there were significant correlations between the ultimate load and BMD in the L4 vertebral body and the midfemur (Fig. 3, Table 2). These results suggest that the increase in bone strength is mainly due to the increase in bone mass by single and combined treatments with alendronate and $1\alpha(OH)D_3$.

Micro-CT analysis

Representative pictures of micro-CT images of the distal metaphysis and the midportion of the femur, and those of the L2 vertebral body are shown as Fig. 4. OVX decreased the trabecular bones of the proximal femur (Fig. 4A) and

L4 vertebral body

All groups

Ultimate Load= $786.8 \times BMD^{0.92}$
$R^2 = 0.177$

ALN-treated groups

UltimateLoad= $1107.2 \times BMD^{1.08}$
$R^2 = 0.426$

1 α -treated groups

Ultimate Load= $691.1 \times BMD^{0.79}$
$R^2 = 0.181$

Midfemur

All groups

Ultimate Load= $1165.3 \times BMD^{1.58}$
$R^2 = 0.664$

ALN-treated groups

Ultimate Load= $761.9 \times BMD^{1.28}$
$R^2 = 0.517$

1 α -treated groups

Ultimate Load= $899.4 \times BMD^{1.41}$
$R^2 = 0.588$

Fig. 3. Relationship between the bone strength and bone mass of the L4 vertebral body and the midfemur in all groups, alendronate-treated groups (ALN-L, ALN-H, ALN-L + 1α and ALN-H + 1α) and 1α(OH)D$_3$-treated groups (1α, ALN-L + 1α and ALN-H + 1α). The ultimate loads and BMDs of the L4 vertebral body and the midfemur were measured as described in Materials and Methods. The correlation is expressed as the equation on each graph. The values of the regression slope (*a*), correlation coefficient (*r*) and correlation significance (*P*) are described in Table 2.

**Table 2.** Regression analysis of ultimate load of the L4 vertebral body and midfemur as a function of BMD

Group	n	L4 vertebral body			Midfemur		
		Regression slope (*a*)	Correlation coefficient (*r*)	Significance (*P*)	Regression slope (*a*)	Correlation coefficient (*r*)	Significance (*P*)
All groups	66	0.92	0.421	0.004	1.58	0.815	<0.001
Sham-OVX groups	22	1.11	0.446	0.038	1.89	0.904	<0.001
Alendronate-treated groups	35	1.08	0.653	<0.001	1.28	0.719	<0.001
1α-treated groups	27	0.79	0.426	0.027	1.41	0.767	<0.001

the vertebral body (Fig. 4C), and the cortical bone of the midfemur (Fig. 4B). All single and combined treatments increased the trabecular and cortical bones. The density of the trabecular bones, but not that of the cortical bones, of combined treatment groups appeared to be higher than that of single treatment groups, showing good accordance with the BMD data in Fig. 1.

Histological analysis

Histomorphometric analysis of the L2 vertebral body and proximal tibia revealed that the bone volume (BV/TV) was significantly decreased by OVX in both bones
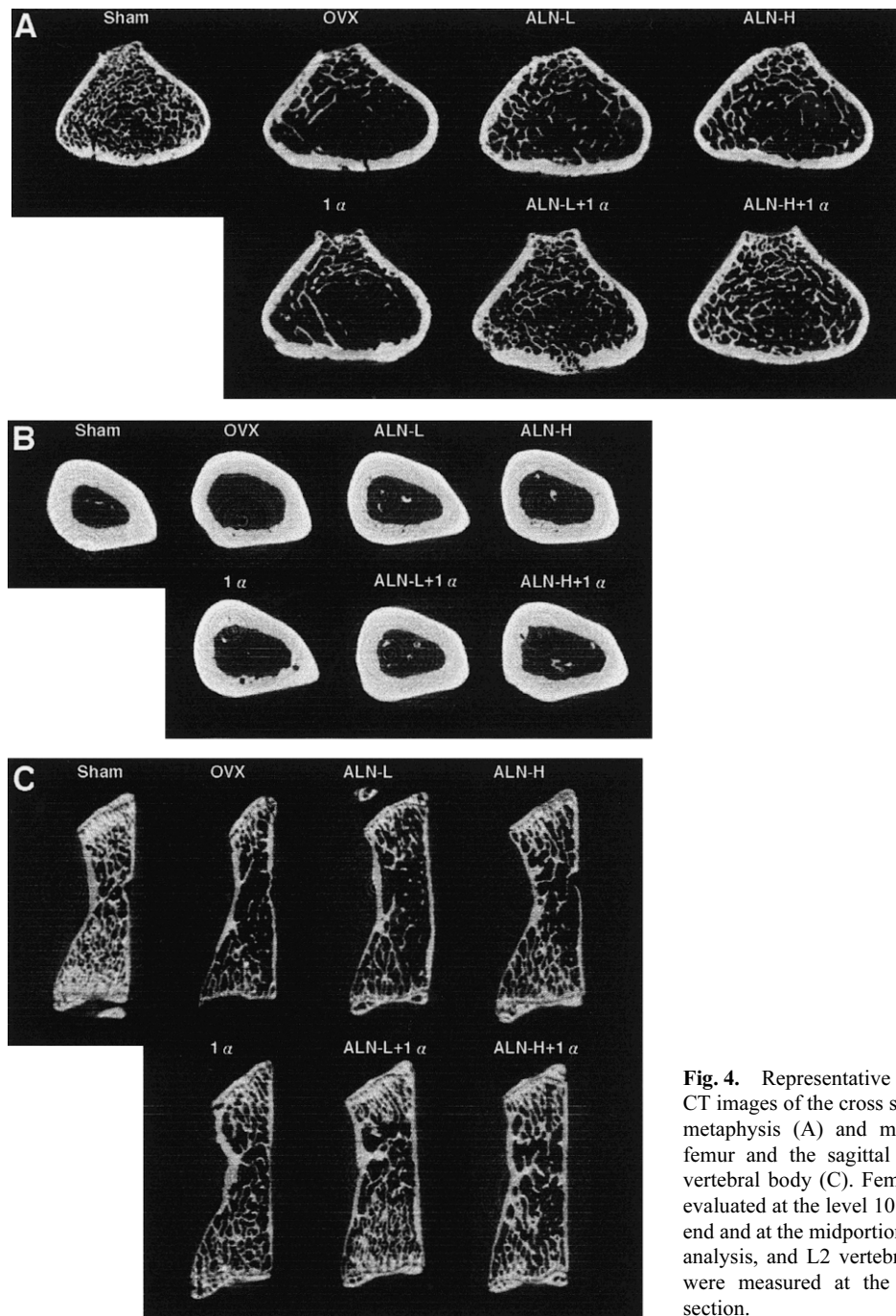
Fig. 4. Representative features of micro-CT images of the cross sections of proximal metaphysis (A) and midshaft (B) of the femur and the sagittal section of the L2 vertebral body (C). Femur specimens were evaluated at the level 10 mm from the distal end and at the midportion by cross-sectional analysis, and L2 vertebral body specimens were measured at the center by sagittal section.

(Table 3). Single treatments caused a slight, but not significant, increase in BV/TV. The treatment with ALN-H + 1α showed a significant increase in BV/TV as compared not only to OVX but also to single treatments (ALN-L and 1α) in the L2 vertebral body. These changes of BV/TV by OVX and the combined treatment were associated with the trabecular number (Tb.N) rather than its thickness (Tb.Th). Bone formation rates (BFR/BS) were increased by OVX in both bones, indicating a high turnover state. This effect

was reduced by all single and combined treatments, significantly in the L2 vertebral body, but not in the proximal tibia. This suppressive effect was prominently seen by treatments with alendronate, especially in the combined treatments.

Representative pictures of von Kossa staining of the proximal tibia are shown as Fig. 5. In this analysis as well, all the single and combined treatments increased the trabecular bone volume and the combined treatments were more

Table 3. Effects on bone histomorphometric indices

	BV/TV (%)	Tb.Th (μm)	Tb.N (/mm)	MAR (μm/day)	BFR/BS (μm^3/μm^2 per day)
L2 vertebral body					
Sham	30.77 ± 4.73^a	53.99 ± 7.83	5.70 ± 0.31^a	0.89 ± 0.19 (6)	2.01 ± 0.98 (6)^a
OVX	20.33 ± 3.75	54.10 ± 5.85	3.76 ± 0.58	1.04 ± 0.09 (6)	9.11 ± 1.33 (6)
ALN-L	22.35 ± 2.64	56.40 ± 4.09	3.96 ± 0.37	1.01 ± 0.14 (6)	4.32 ± 1.62 (6)^b
ALN-H	23.68 ± 2.77	54.53 ± 9.85	4.40 ± 0.46	0.82 ± 0.18 (4)	1.23 ± 0.75 (4)^b
1α	22.25 ± 1.70	54.64 ± 3.10	4.08 ± 0.28	0.94 ± 0.11	4.65 ± 2.39^{bd}
ALN-L + 1α	22.82 ± 2.95	52.39 ± 8.99	4.42 ± 0.64	0.88 ± 0.38 (3)	1.71 ± 1.42 (3)^b
ALN-H + 1α	27.42 ± 3.36^{bce}	59.48 ± 8.83	4.66 ± 0.62^b	0.92 ± 0.11 (3)	0.56 ± 0.35 (3)^{bce}
Proximal tibia					
Sham	18.08 ± 4.50^a	36.14 ± 7.56	4.99 ± 0.55^a	1.00 ± 0.21	9.02 ± 3.42 (6)^a
OVX	3.22 ± 1.19	36.42 ± 4.54	0.87 ± 0.25	1.00 ± 0.08	20.03 ± 7.37
ALN-L	5.72 ± 2.27	38.51 ± 5.72	1.49 ± 0.59	0.85 ± 0.09	13.45 ± 7.43
ALN-H	5.89 ± 2.49	35.61 ± 4.74	1.66 ± 0.71	1.08 ± 0.25 (3)	9.78 ± 3.26 (3)
1α	4.27 ± 2.58	37.01 ± 6.95	1.13 ± 0.62	0.89 ± 0.13	15.35 ± 7.20
ALN-L + 1α	6.69 ± 2.75	36.12 ± 3.43	1.86 ± 0.76	0.84 ± 0.22 (3)	11.69 ± 10.08 (3)
ALN-H + 1α	8.56 ± 5.38^b	38.50 ± 4.96	2.13 ± 1.24^b	0.78 ± 0.26 (2)	8.38 ± 10.17 (2)

BV/TV, bone volume / tissue volume; Tb.Th, trabecular thickness; Tb.N, trabecular number; MAR, mineral apposition rate; BFR/BS, bone formation rate / bone surface. [a]$P<0.05$ vs OVX group (Student's two-tailed t-test). [b,c,d,e]$P<0.05$ vs OVX, ALN-L, ALN-H, 1α groups, respectively (Tukey's test). Data are expressed as the mean $\pm$ S.D. n = 7, unless otherwise indicated in parentheses.

effective than the single treatments. In addition, it was confirmed that these treatments including alendronate did not impair the mineralization as determined by the growth plate thickness.

DISCUSSION

Our results demonstrated that the effects of combined treatments with alendronate and $1\alpha(OH)D_3$ on the mass and strength of trabecular and cortical bones were greater than those of single treatments. Because the increase in the mechanical strength was proportional to that in BMD both in the trabecular and cortical bones, the stimulatory effects of these treatments on bone strength are ascribable primarily to the effects on bone mass.

We aimed at clinical application of the combination of bisphosphonates and vitamin D derivatives as a curative, rather than preventive, treatment of established postmenopausal osteoporosis. The present study therefore differed from our previous study (34) which showed a preventive effect of the combination of etidronate and $1\alpha(OH)D_3$ on bone loss starting 1 week after OVX in 14-week-old rats. In the present study we ovariectomized 40-week-old rats in whom bone growth and maturation have been completed (35, 36), and treatment started 15 weeks after surgery. As a bisphosphonate, this study used alendronate, a more potent and prevalent bisphosphonate than etidronate.

Since the present study was not designed to elucidate the mechanism of action of the combined treatment, the precise cellular mechanism of its action remains unclear. However, the analysis of plasma and urine biochemistry revealed that $1\alpha(OH)D_3$, but not alendronate, significantly increased the urinary calcium level. On the other hand, neither plasma osteocalcin nor urinary deoxypyridinoline level was affected by $1\alpha(OH)D_3$, but both were decreased by alendronate dose-dependently. These findings may imply a difference in functions of these two agents in restoring bone loss in OVX rats: $1\alpha(OH)D_3$ may enhance calcium absorption from the gastrointestinal tract, while alendronate decreases bone turnover. $1,25(OH)_2D_3$, the active form from $1\alpha(OH)D_3$, is known to stimulate bone formation directly and bone resorption indirectly by acting on osteoblastic cells (20, 50). It may be that alendronate blocks the catabolic action, but not the anabolic action, of $1\alpha(OH)D_3$ in the combined treatment. In fact, a previous study showed that combined alendronate and prostaglandin E$_2$ (PGE$_2$) treatment prevented the resorption induced by PGE$_2$ but not the stimulation of bone formation on endocortical and periosteal surfaces and resulted in a significant increase in cortical thickness in OVX rats (51). However, combined treatments with bisphosphonates and other anabolic agents do not always lead to beneficial effects on bone. Combined treatment with tiludronate and PTH is reported to reduce bone mass in elderly female sheep, although its mechanism
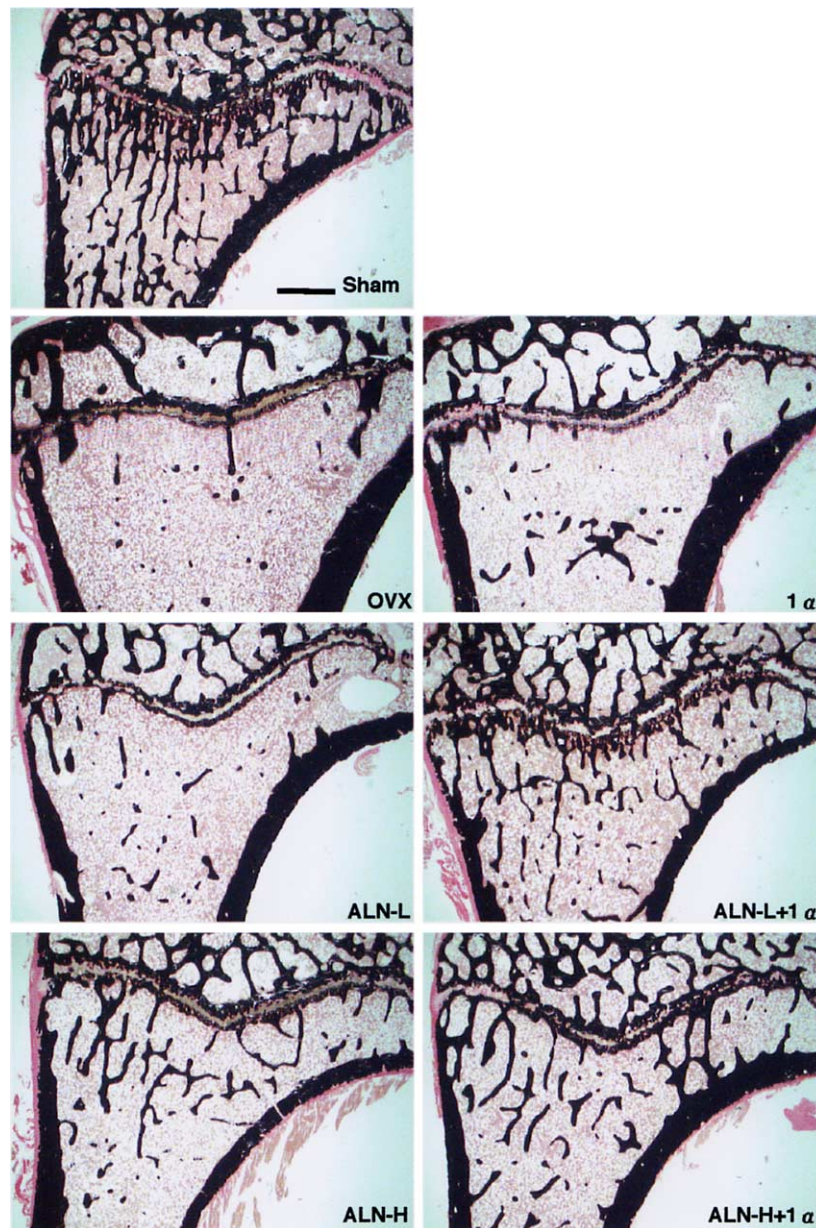
Fig. 5. Representative histological features of the proximal tibiae. Tibiae were dehydrated through graded alcohol and xylene, and stained with von Kossa. Bar indicates 1 mm.

is unclear (52). The possible mechanism of action of each agent should at least be taken into consideration when combined therapy is applied for clinical use.

Although several previous studies demonstrated that a single treatment with $1\alpha(OH)D_3$ significantly increased bone mass in OVX rats (30 – 32, 34), our study failed to show significant anabolic actions of the treatment except on the midfemur BMD. Because this study was planned aiming at clinical application, we employed the dose of 0.02 μg/kg per day, which is equivalent to that used clinically (0.5 – 1.0 μg/day). Previous studies showing signifi-

cant anabolic actions used much higher doses (0.1 – 0.25 μg/kg per day) and were accompanied by hypercalcemia, while this study showed only hypercalciuria without hypercalcemia as usually seen in patients treated with $1\alpha(OH)D_3$. The doses of $1\alpha(OH)D_3$ used in the previous preventive study on the combined treatments with etidronate and $1\alpha(OH)D_3$ were 0.1 and 0.2 μg/kg per day (34), and caused severe hypercalcemia and hyperphosphatemia, although the anabolic effects of the single and combined treatments seemed stronger than those in the present study. Since the combined treatment groups with

bisphosphonates and $1\alpha(OH)D_3$ showed lower calcium level than the single treatment group of $1\alpha(OH)D_3$ in both the present and previous studies (34), the combination with bisphosphonates may prevent the increase in the plasma calcium level caused by $1\alpha(OH)D_3$ alone. This study employed a chow containing 1.18% (w/w) calcium, which is relatively higher than the physiological calcium intake level. Hence, these results suggest that even in patients who are being treated with calcium supplement, the combination of alendronate and $1\alpha(OH)D_3$ will reduce the risk of hypercalcemia.

In this study, we employed the two doses of alendronate: 0.2 and 1.0 mg/kg per day, which are equivalent to or relatively higher than those used clinically (5 – 10 mg/day). These two doses were also determined to span the boundary dose to show a significant ameliorative effect on OVX-induced bone loss based on our previous study (11). All analyses in this study revealed the dose-dependent anabolic effects of the single treatment of alendronate; however, they were much weaker than those reported in previous clinical studies (53, 54) and only the high dose of alendronate showed significant stimulation of bone strength. Since the gastrointestinal absorption of bisphosphonates is very poor, especially in the presence of calcium, this relatively weak effect may be due to the paucity of alendronate absorption in rats that cannot be treated in a fasting condition like humans. In previous studies except ours (11) that showed an anabolic effect of alendronate on bone loss in OVX animals, this agent has been administered by i.v. or s.c. injection (12, 13, 55, 56).

Taken together, our results demonstrate for the first time that the combination of daily alendronate and $1\alpha(OH)D_3$ is promising for the treatment of established postmenopausal osteoporosis. In fact, a clinical study revealed that a combined treatment with alendronate and $1,25(OH)_2D_3$ is more effective than treatment with alendronate or $1,25(OH)_2D_3$ alone in terms of changes in BMD at the spine and femoral neck of postmenopausal women (57). A combination of intermittent cyclical etidronate and daily $1,25(OH)_2D_3$ is also reported to be more beneficial for bone mass than cyclical etidronate alone in these women (58). We thus conclude that a combination of bisphosphonates and vitamin D derivatives may be useful in prevention and treatment of postmenopausal osteoporosis.

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