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A Comparison of Alfacalcidol and Menatetrenone for the Treatment of Bone Loss in an Ovariectomized Rat Model of Osteoporosis

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Abstract. We conducted this study to evaluate the characteristic effects of alfacalcidol (ALF) and menatetrenone (VK) in preventing bone loss using a ovariectomized rat model of osteoporosis. Bilateral ovariectomy (OVX) or sham operation was performed on 10-month-old female Wistar rats. OVX caused a significant decrease in the bone mass and the mechanical strength of the lumbar vertebra as well as the femur 6 months after surgery. VK treatment (30 mg/kg, food intake) required a 6-month period to prevent the bone loss induced by estrogen deficiency, whereas ALF (0.1 or 0.2 μg/kg, p.o.) increased the bone mass and the mechanical strength of the lumbar vertebra as well as the femur in a 3-month treatment period, far above the level in the sham-operated rats. Neither ALF or VK caused hypercalcemia, despite administration for as long as 6 months. By doing a micro-CT analysis of the vertebral trabecular microstructure, it was revealed that ALF treatment increased the interconnections and the plate-like structures and that VK significantly increased the trabecular number. It was also indicated that the increase in spinal strength by ALF treatment was closely associated with improvement of the microstructure, but not VK. The results of histomorphometric analysis showed that ALF caused a significant suppression of bone resorption yet maintained formation in the endocortical perimeter, and also stimulated bone formation in the periosteal perimeter, thereby causing an increase in cortical area. No marked effect of VK on histomorphometric parameters was observed, whereas VK as well as ALF maintained the material strength at femoral midshaft of the normal level, suggesting that VK affected bone quality and thereby prevented the decrease in mechanical strength of femur caused by OVX. In conclusion, it was demonstrated that the two drugs, ALF and VK, differed markedly in their potency and mechanisms for improving bone strength. These results have important implications in understanding the characteristic actions of vitamin K and active vitamin D on bone metabolism.

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In recent years, as the elderly population increases, the number of patients with osteoporosis has been increasing. Osteoporosis is a degenerative disease characterized by reduced bone mass and microarchitectural deterioration of bone, leading to enhanced bone fragility and a consequent increase in fracture risk [1]. To reduce the frequency of fracture, it is important to improve bone strength clinically using the optimal drug therapy. It has been reported that decrease in the mechanical strength of bone is not only caused by bone loss but also by changes in bone microstructure [2, 3]. In addition, the quality of bone has recently been considered as a factor associated with fractures [4].

Activated vitamin D_3(1α, $25(OH)_2D_3$) is one of the hormones regulating calcium metabolism. Its prodrug, alfacalcidol (1α (OH) D_3: ALF), has been used in certain countries including Japan, for the treatment of a variety of metabolic bone diseases such as rickets/osteomalacia, renal osteodystrophy, and osteoporosis [5, 6]. We had already reported that, in the vitamin D-sufficient ovariectomized (OVX) rat model of osteoporosis, ALF treatment markedly suppressed osteoclastic bone resorption while maintaining or even stimulating bone formation, and consequently increased bone mass with a parallel improvement in the mechanical strength of bone, thus confirming its pharmacological efficacy [7, 8].

Since vitamin K is essential for the γ-carboxylation of glutamic acid residues of osteocalcin, a noncollagenous protein present in bone, this vitamin is thought to play a role in bone metabolism through the γ-carboxylation of osteocalcin [9]. The clinical efficacy of vitamin K therapy for osteoporosis has also been reported [10]. In elderly patients with femoral neck fracture, the serum carboxylated osteocalcin level, the carboxylation activity of the osteocalcin molecule, and the serum vitamin K level are lower than in elderly persons without fractures [11, 12]. Comparing the relative molar activity of several forms of vitamin K in terms of γ-carboxylation in the blood coagulation system, vitamin K_2 with 4 isoprene

units (menatetrenone: VK) was the most potent [13]. It has been reported that VK prevents bone loss in OVX animal models [14, 15] and steroid-induced osteopathy [16]. It has also been reported that VK inhibits bone resorption [17, 18] and stimulates bone mineralization by increasing the production of osteocalcin *in vitro* [19].

Although it is assumed that both ALF and VK play essential roles in bone homeostasis, the differences among their actions *in vivo* are not fully understood. In order to achieve more pertinent clinical use of these agents, their individual pharmacological effects and the differences in their action *in vivo* should be clarified. In the present study, we examined the preventive effects of ALF and VK on bone loss and fragility in the OVX rat model of osteoporosis, and evaluated their potency and characteristic effects for improving bone strength, using micro-CT and histomorphometric analysis.

Materials and methods

Animals

Female Wistar-Imamichi rats, at 10 months of age, were purchased from Imamichi Institute for Animal Reproduction (Ibaraki, Japan) and acclimated under standard laboratory conditions at 24 ± 2°C and 50–60% humidity. The rats were allowed free access to tap water and commercial standard rodent chow (CE-2) containing 1.25% calcium, 1.06% phosphate, and 2.0 IU/g of vitamin D_3 (CLEA Japan, Inc., Tokyo). Body weight and food intake were measured once a week.

Experimental design

At 10 months of age, 85 rats were randomly assigned to 12 groups. The rats in one group were sacrificed at day 0 (baseline control). The animals in two groups were sham-operated, and others underwent bilateral ovariectomy (OVX). The OVX rats were further divided into 9 groups 2 weeks after the surgery. In the sham and OVX control groups, rats received the vehicle (MCT) orally at a dose of 1 ml/kg body weight (BW) five times a week.

Alfacalcidol (1α(OH)D_3: ALF) was given orally at 0.1 and 0.2 μg/kg BW five times a week, and menatetrenone (VK) was given at 3 and 30 mg/kg BW per day as a dietary supplement. ALF was synthesized at Chugai Pharmaceutical Co., Ltd. (Tokyo, Japan), dissolved in a vehicle (medium chain triglyceride: MCT), and diluted to a given concentration. The stock solutions were protected from light and stored at 4°C. VK was purchased from Sigma-Aldrich, Inc. (St. Louis, USA). To examine whether the expected dose of VK had been taken, VK intake from the diet was calculated from the data for body weight and food intake measurements. Thus, VK intake was adjusted to a previously determined amount throughout the experimental period. The treatment was continued for 12 or 24 weeks. These animal studies were carried out in accordance with Chugai Pharmaceutical's ethical guidelines for animal care, and the experimental protocols were approved by the animal care committee of the institution.

Bone labeling by subcutaneous injection of tetracycline (20 mg/kg BW) and calcein (8 mg/kg BW) was performed at 13 and 3 days, respectively, prior to sacrifice. Urine was collected during the final 24 h of fasting. Blood was collected from the abdominal aorta under ether anesthesia and centrifuged to obtain the serum. Urine and serum samples were stored at -80°C until assay. The lumbar vertebrae and bilateral femurs were removed. The fifth lumbar vertebra (L5) was separated and stored with the left femur at -80°C for assessment of mechanical strength. The other vertebrae (L2–L4) and the right femur were stored in 70% ethanol for the measurement of bone mineral density (BMD).

Biochemical Analysis

Serum calcium (Ca), inorganic phosphorus (Pi) concentrations, and alkaline phosphatase activity (ALP) were measured using an autoanalyzer (Hitachi 7070; Hitachi Co., Ltd., Tokyo, Japan). Urinary Ca, Pi, and creatinine (Cre) were measured by the autoanalyzer. Urinary deoxypyridinoline (D-Pyr) was measured by a YRILINKS-D assay kit (Metra Biosystems, Inc., California, USA), and the data were corrected for urinary Cre concentration.

Measurement of Bone Mineral Density

The bone mineral density (BMD, mg/cm^2) of the lumbar vertebrae (L2–L4) and the right femur was measured by dual-energy X-ray absorptiometry (DCS-600; Aloka Co., Ltd., Tokyo, Japan) using the small-animal scan mode.

Measurement of Mechanical Properties

Compression Test for L5 Vertebra. The mechanical strength of L5 was measured by the compression test [20]. The specimen was fixed with a clamp at the bases of the transverse processes, in the holder of a diamond band saw. By removing the cranial and caudal ends of the specimen, the planoparallel surfaces were obtained for a compression test. From each vertebral body, a central cylinder with planoparallel ends and a height of approximately 5 mm was obtained. The cylinder samples were placed centrally on the smooth surface of a steel disk attached to a load tester (Tensilon UTA-1T; Orientec Co., Ltd., Tokyo, Japan). A compression force was applied in a cranio-caudal direction to the specimen with a steel disk at a deformation rate of 2 mm/min. The ultimate compressive load (N) was obtained as the mechanical strength directly from the load-deformation curve.

Three-point Bending Test for Femoral Midshaft. The mechanical strength of the left fermur was measured by the three-point bending test [21] using the tester (Tensilon UTA-1T; Orientec, Tokyo, Japan). The left femur was placed on a special holding device with supports located 12 mm apart. The bending force was applied with the cross head at a speed of 10 mm/min, until a fracture occurred. From the load-deformation curve, the breaking strength (N) was obtained as the mechanical strength. The maximum material strength (Ms: normalized bending strength, N/mm^2) was calculated from the following equation:

Ms = M/Z where M (the bending moment to failure, N · mm) = 1/4 × ultimate load (N) × L (mm), Z (the modulus of section, mm^3) = 2 × I/D, L (mm) is the distance between the supports of the holding device (12 mm), I (mm^4) is the moment of inertia, D is the antero-posterior outer diameter (mm) [22].

Structural Analysis Using Micro-computed Tomography (mCT)

The micro-CT apparatus (μCT20) and the analyzing software used in this study were obtained from SCANCO Medical AG (Bassersdorf, Switzerland) [23]. The system of this micro-CT has a micro X-ray source (10 μm, 25 keV) directed toward the

sample. The quantitative modification of the X-ray beam by apatite crystals contained in the bone sample is then analyzed by a plane detector (CCD array; 1024 elements). The process is piloted by a DEC a-station (Digital Equipment Corp., Marseille, France), and an open VMS system in cluster configuration to perform the 3D analysis. The whole spinal body (L5) was scanned in 250 slices, each 13 μm thick in the dorsoventral direction.

On the original 3D images, morphometric indices were directly determined from the binalized volume of interest (VOI) [24]. Three-dimensional reconstruction of bone was performed using the triangulation algorithm. The volume of trabecular bone (BV, mm^3) was calculated with tetrahedrons corresponding to the enclosed volume of the triangulated surface. The total tissue volume (TV, mm^3) is the volume of the whole examined sample. To be able to compare samples with different sizes, these were normalized such as the bone volume fraction (BV/TV, %). Using the original application, the histomorphometric parameters, which were the trabecular number (Tb.N, 1/mm), the trabecular thickness (Tb.Th, μm), and the trabecular separation (Tb.Sp, μm), were directly measured on 3D images using the method described by Hildebrand et al. [25], not using the parallel plate model. Thus, the parameters of Tb.Th, Tb.Sp, and Tb.N are model-independent indices, not biased by eventual deviations of the actual structure. For the nonmetric parameters, the trabecular bone pattern factor (TBPf) and the structure model index (SMI) were computed using software provided with the micro-CT machine. The trabecular bone pattern factor (TBPf, 1/mm), representing the ratio of concave to convex surfaces in 2D sections of trabeculae, was measured for each slice, and the mean value was determined for each specimen [26]. The structure model index (SMI) is a parameter to quantify the characteristic form of a three-dimensional structure of plates and rods [27].

Bone Histomorphometry

The diaphysis of the right femur was fixed in 70% ethanol and stained according to the method of Villanueva [28]. After dehydration with ethanol and acetone, the sample was embedded in methyl methacrylate (Wako Pure Chemical Industries, Ltd., Osaka, Japan). For the femoral diaphysis, 20–30 μm thick cross-cut ground sections were obtained with a micro-grinding machine system (KG4000; EXAKT Apparetebau GmbH and Co. KG, Norderstedt, Germany) and prepared for measurement. The image of the specimen, observed under a fluorescence microscope and recorded with a video camera, was processed using a plotter (Cosmozome 1SA; Nikon Corp., Tokyo, Japan) to measure the total cross-sectional areas (mm^2), the marrow area (mm^2), the cortical bone area (mm^2), the outer perimeter (mm), and the inner perimeter (mm). Kinetic parameters such as the eroded surface (Es, μm), the osteoclast number (N.Oc), the single-labeled surface area (sLS, μm), the double-labeled surface area (dLS, μm), and the mineralizing surface area (MS, μm) were obtained in the perimeters of femoral diaphysis. From these primary parameters, the following parameters were calculated: the eroded surface area (ES/BS, %), the number of osteoclast per mm trabecular surface (N.Oc/BS, /mm), the bone formation rate (BFR/BS, $\mu m^3/\mu m^2$/year), and the mineralizing surface (MS/BS, %). Osteoclasts were identified as cells that form resorption lacunae at the bone surface. Nomenclature, symbols, and units used in this study are those described in the Report of the American Society for Bone and Mineral Research Nomenclature Committee [29].

Statistical Analysis

All data were expressed as the mean ± standard error of the mean (SEM). Statistical analysis was carried out by an analysis of variance (ANOVA) using Statistic Analysis System (SAS) software. The significance of differences was determined using Dunnett's multiple test (for comparison with OVX-vehicle group) or Student's *t*-test (Sham-operated group vs. OVX-vehicle group). Using a simple regression analysis, correlation coefficients were obtained. A P value less than 0.05 was considered a significant difference.

Results

Body Weight and Biochemical Parameters (Table 1)

OVX caused an increase in the body weight over the experimental period. In the ALF 0.2 μg/kg-treated groups, the body weight was significantly lower than in the OVX group, which was at the same level as that in the sham-operated groups. VK did not affect the body weight gain after OVX.

With the doses used in the current study, ALF did not raise the serum Ca levels significantly above those in the sham-operated groups. The serum Pi levels in the ALF-treated groups were significantly higher than in the sham-operated groups. In the VK-treated groups, the serum Ca and Pi levels showed no marked changes. The serum ALP level in the OVX group increased significantly compared with that in the sham-operated group 6 months after surgery. Neither ALF nor VK markedly affected the ALP levels in OVX rats.

The values of urinary Ca/Cre in the ALF-treated groups were significantly increased compared with those of the OVX groups. In the VK-treated groups, no marked change was observed in the urinary Ca levels. OVX caused an increase in the urinary D-Pyr excretion 3 months after surgery, and the difference was significant compared with the sham-operated group. The values of urinary D-Pyr/Cre in the groups treated with ALF for 3 months were significantly decreased compared with that in the OVX group. The D-Pyr/Cre levels in the group treated with VK at 30 mg/kg for 6 months were lower than in the OVX group, but the difference was not significant.

Serial Changes in Bone Mineral Density of Lumbar Vertebra and Femur (Fig. 1A, B)

In the sham-operated group, the BMD values of the lumbar vertebra (Fig. 1A) and the femur (Fig. 1B) did not show a marked change throughout the study period of 6 months. In the OVX control group, BMD at both sites decreased progressively and was significantly lower than that in the sham-operated rats 6 months after surgery (Fig. 1A, B). In the groups treated with ALF for 3 months, regardless of dosage administered, the BMD values of the lumbar vertebra (Fig. 1A) as well as the femur (Fig. 1B) were significantly higher than in the sham-operated group, and increased further at 6 months. The BMD values at both sites in the group treated with VK at 30 mg/kg for 6 months were significantly higher than in the OVX control group (Fig. 1A, B).

Table 1. Body weight and biochemical parameters in OVX rats treated with alfacalcidol or menatetrenone

		Serum			Urine	
Group	Body Weight (g)	Calcium (mg/dL)	Phosphorus (mg/dL)	Alkaline phosphatase (IU/L)	Ca/Cre	D-Pyr/Cre (nM/mM)
10-month-old						
Basal control (Intact)	374 ± 7	9.8 ± 0.08	5.7 ± 0.45	279 ± 39.6	0.13 ± 0.03	16.0 ± 1.35
13-month-old (3-month treatment)						
Sham	375 ± 16	10.1 ± $0.14^{\dagger\dagger}$	5.4 ± 0.21	328 ± 39.6	0.09 ± 0.02	17.1 ± 2.08
OVX + vehicle	443 ± 13^{**}	9.8 ± 0.03	5.3 ± 0.31	413 ± 43.1	0.09 ± 0.02	31.4 ± 2.24^{***}
OVX + ALF 0.1 μg/kg	429 ± 9^{**}	10.3 ± $0.05^{\dagger\dagger\dagger}$	6.6 ± $0.30^{\dagger *}$	434 ± 41.8	0.55 ± $0.05^{\dagger\dagger\dagger ***}$	15.2 ± $1.35^{\dagger\dagger\dagger}$
OVX + ALF 0.2 μg/kg	404 ± $7^{\dagger}$	10.4 ± $0.08^{\dagger\dagger\dagger}$	6.9 ± $0.40^{\dagger\dagger **}$	408 ± 57.5	0.69 ± $0.06^{\dagger\dagger\dagger ***}$	13.2 ± $1.01^{\dagger\dagger\dagger}$
OVX + VK 30 mg/kg	445 ± 8^{**}	10.1 ± $0.12^{\dagger}$	5.4 ± 0.33	446 ± 63.9	0.11 ± 0.03	39.4 ± 3.83^{***}
16-month-old (6-month treatment)						
Sham	401 ± 12	10.0 ± 0.23	5.0 ± 0.54	219 ± 31.0	0.09 ± 0.04	15.3 ± 1.43
OVX + vehicle	491 ± 13^{**}	95 ± 0.09	5.1 ± 0.25	439 ± 26.4^{***}	0.03 ± 0.01	35.6 ± 242^{***}
OVX + ALF 0.1 μg/kg	445 ± 13	10.5 ± $0.18^{\dagger\dagger\dagger}$	7.4 ± $0.26^{\dagger\dagger\dagger ***}$	378 ± 33.7^{*}	0.25 ± $0.07^{\dagger}$	14.8 ± $1.03^{\dagger\dagger\dagger}$
OVX + ALF 0.2 μg/kg	419 ± $9^{\dagger\dagger}$	10.3 ± $0.09^{\dagger\dagger\dagger}$	6.5 ± $0.28^{\dagger\dagger *}$	335 ± 49.1	0.34 ± $0.06^{\dagger\dagger\dagger *}$	13.4 ± $1.11^{\dagger\dagger\dagger}$
OVX + VK 3 mg/kg	477 ± 9^{**}	9.6 ± 0.11	5.4 ± 0.22	478 ± 92.7^{*}	0.04 ± 0.01	36.9 ± 2.53^{***}
OVX + VK 30 mg/kg	477 ± 9^{**}	9.6 ± 0.08	5.5 ± 0.31	371 ± 55.8	0.03 ± 0.01	26.9 ± 2.30^{**}

Each value represents mean ± SEM (n = 6–8)

$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Sham control group

$^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ vs. OVX control group

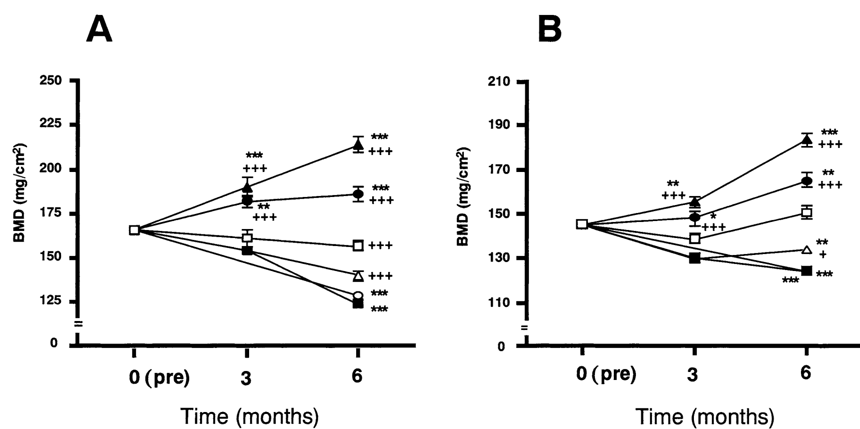

Fig. 1. Changes in bone mineral density of the 2nd–4th lumbar vertebra (**A**) and the femur (**B**) in ovariectomized (OVX) rats treated with alfacalcidol or menatetrenone. Each value represents the mean ± SEM (n = 6–8). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the sham-operated group at the same time point. $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ compared with the OVX-control group at the same time point. □: sham-operated group, ■: OVX-control group, ●: ALF 0.1 μg/kg-treated group, ▲: ALF 0.2 μg/kg-treated group, ◯ :VK3 mg/kg-treated group, △: VK30 mg/kg-treated group.

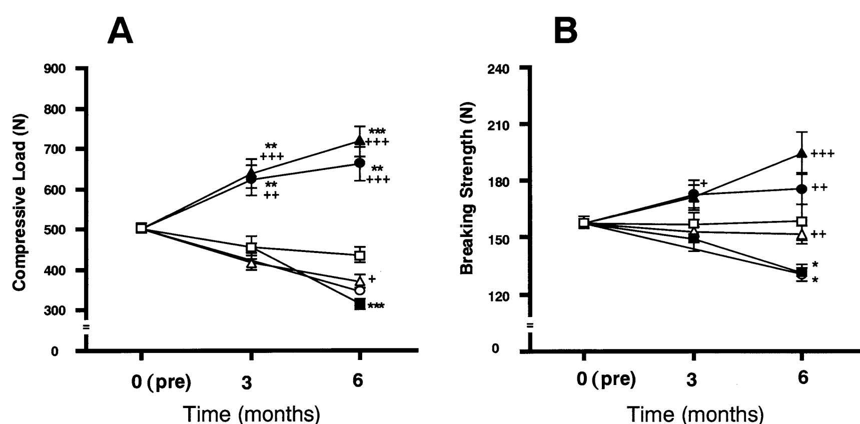

Fig. 2. Changes in mechanical strength of the 5th lumbar vertebra (**A**) and the femoral diaphysis (**B**) in ovariectomized (OVX) rats treated with alfacalcidol or menatetrenone. Each value represents the mean ± SEM (n = 6–8). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with the sham-operated group at the same time point. $^{+}P < 0.05$, $^{++}P < 0.01$, $^{+++}P < 0.001$ compared with the OVX-control group at the same time point. □: sham-operated group, ■: OVX-control group, ●: ALF 0.1 μg/kg-treated group, △: ALF 0.2 μg/kg-treated group, ◯: VK3 mg/kg-treated group, ▲: VK30 mg/kg-treated group.

Serial Changes in Mechanical Strength of Lumbar Vertebral Body and Femoral Mid-shaft (Fig. 2A, B)

In the sham-operated groups, the compressive load of the lumbar vertebra and the breaking strength of the femur showed no marked change throughout the study period (Fig. 2A, B). In the OVX control group, however, the mechanical strength of the lumbar vertebra was significantly lower than in the sham-operated group 6 months after surgery (Fig. 2A), and this trend was also pronounced on the femur (Fig. 2B). In the groups treated with ALF for 3 months, the load values of the lumbar vertebra were significantly higher than in the sham-operated group, and they increased further at 6 months (Fig. 2A). At the femur, the breaking strength of the mid-shaft in the group treated with ALF at a dose of 0.2 μg/kg for 3 months was significantly higher than that in the OVX control group, and in the group treated with ALF for 6 months, regardless of dosage administered, the values of femoral strength were significantly higher than that in the OVX control group (Fig. 2B). The values of mechanical strength in the lumbar vertebra as well as the femur were significantly higher in the group treated with VK at a dose of 30 mg/kg for 6 months compared with the OVX control group, but these values were lower than the level in the sham-operated group (Fig. 2A, B).

Effects of Six Months Treatment with Alfacalcidol and Menatetrenone on Bone Microarchitecture — Micro-CT Analysis (Fig. 3, Table 2)

Micro-CT images revealed that the microarchitecture of the lumbar vertebra in the OVX group 6 months after surgery was interrupted with many spaces and showed loss of interconnections, compared with that in the sham-operated group (Fig. 3). In the ALF-treated groups, the interconnections were restored in trabecular bone with an increase of the trabecular plates (Fig. 3). Treatment with VK at 30 mg/kg maintained the trabecular structure at the same level as the sham-operated group.

The BV/TV, the Tb.Th, and the Tb.N in the OVX group were significantly lower than those in the sham-operated group. In the ALF-treated groups, these parameters were increased dose dependently and were significantly larger than those in the sham-operated group (Table 2). In the VK 30 mg/kg-treated group, the Tb.N was the only parameter that significantly increased in comparison with the OVX control group (Table 2). The increase in the Tb.Sp after OVX was suppressed by administration of ALF, and the Tb.Sp in the ALF-treated groups decreased significantly in comparison with the sham-operated group. In the group treated with VK at a dose of 30 mg/kg, the

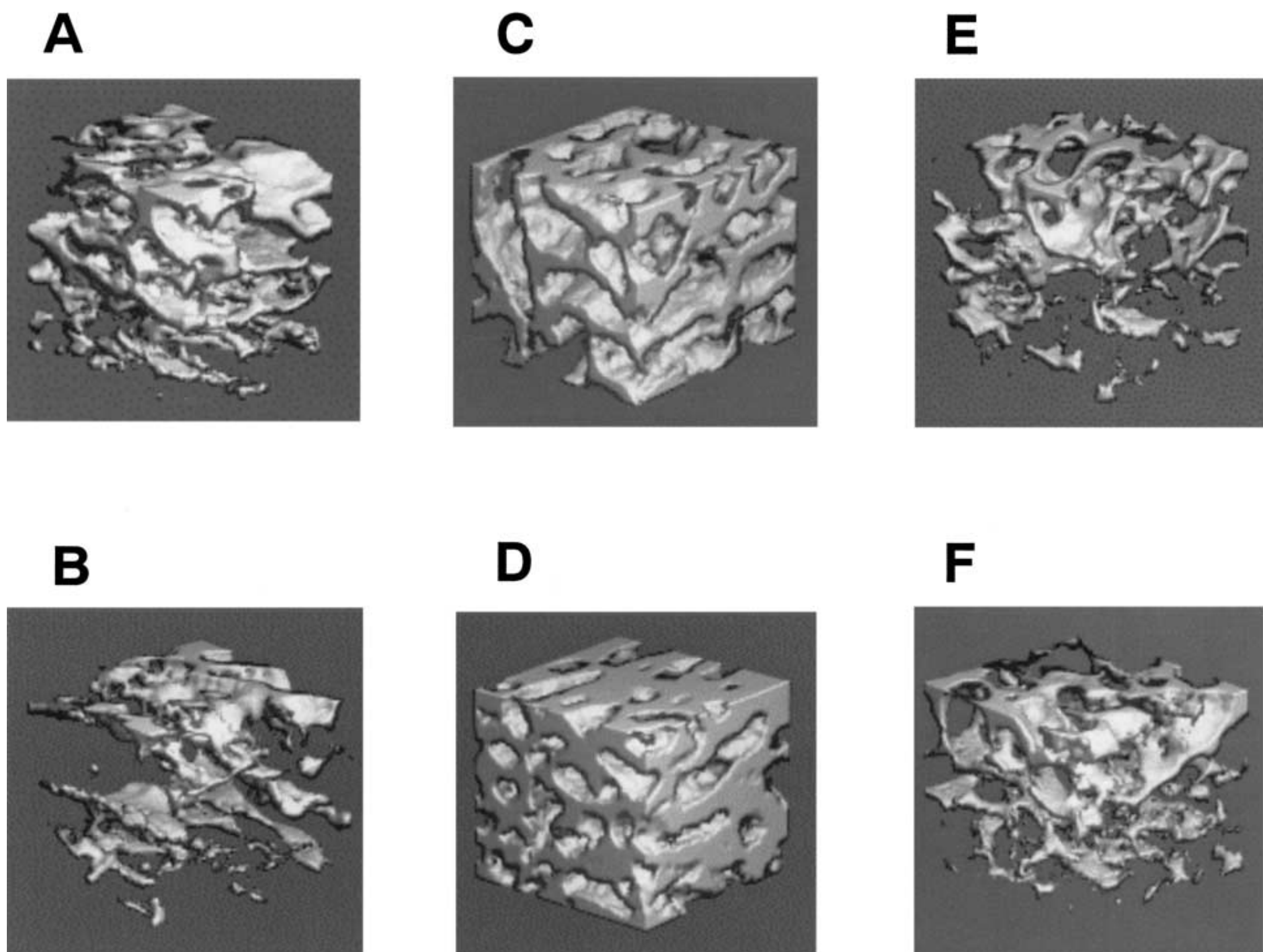

Fig. 3. Three-dimensional trabecular microarchitectural images of rat L5 vertebral body using micro-CT analysis. Rats were bilaterally ovariectomized at 10 months of age. Alfacalcidol was administered orally 5 times a week, and menatetrenone was given as a dietary supplement for 6 months. (**A**) Sham-operated rat, (**B**) OVX-control rat, (**C**) alfacalcidol 0.1 μg/kg-treated rat, (**D**) alfacalcidol 0.2 μg/kg-treated rat, (**E**) menatetrenone 3 mg/kg-treated rat, (**F**) menatetrenone 30 mg/kg-treated rat.

Tb.Sp was significantly lower than that in the OVX group.

The TBPf and the SMI in the OVX group were significantly higher than those in the sham-operated group, indicating that OVX induced a decrease in the connectivity of the trabecular bone. Both parameters significantly decreased in the ALF-treated group compared with those in the sham-operated group (Table 2). These parameters in the VK-treated group were also lower than those in the OVX control group, but the difference was not significant (Table 2).

Relationship between Compressive Load and Structural Parameters in Lumbar Vertebra (Table 3)

In order to assess in more detail the relationship between the effects of ALF and VK on the mechanical strength and the microstructure of the lumbar vertebra, the compressive load values were plotted against the BMD values or the structural indices by a linear regression analysis. In the OVX group, the BMD and the microstructural indices were significantly correlated with the load values. The Tb.N and the TBPf were highly correlated with a reduction in the mechanical strength of the lumbar vertebra after estrogen deficiency (Table 3). In the ALF-treated group, the compressive load values were significantly correlated with the structural parameters, such as the TBPf, as well as the BMD (Table 3). In contrast, in the VK-treated group, the individual structural parameters were minor associated factors with respect to the mechanical strength of the lumbar vertebra, and the Tb.N was the only parameter that correlated with the compressive load value.

Effects of Six Months Treatment with Alfacalcidol and Menatetrenone on Femoral Diaphysis — Histomorphometric Analysis (Table 4)

Cross-sectional morphology In the ALF 0.2 μg/kg-treated group, the total cross-sectional area of the femoral diaphysis was significantly higher than in the sham-operated group (Table 4). In the VK-treated groups, the

Table 2. Effects of alfacalcidol and menatetrenone on microarchitectural indices of the L5 vertebra (6-month treatment)

Group	Structual indices				Nonmetric indices	
	BV/TV (%)	Tb.Th (μm)	Tb.N (/mm)	Tb.Sp (μm)	SMI	TBPf (/mm)
16-month-old (6-month treatment)						
Sham	29.2 ± 1.4	92.5 ± 2.1	4.5 ± 0.08	225.3 ± 4.3	1.09 ± 0.15	−1.47 ± 0.60
OVX + vehicle	19.0 ± 1.3***	85.5 ± 2.6*	3.4 ± 0.08***	292.6 ± 7.1***	1.71 ± 0.11**	2.97 ± 0.62***
OVX + alfacalcidol 0.1 μg/kg	58.0 ± 2.5†††***	140.6 ± 5.6†††***	5.5 ± 0.13†††***	178.7 ± 4.8†††***	−3.67 ± 0.62†††***	−11.34 ± 1.08†††***
OVX + alfacalcidol 0.2 μg/kg	69.7 ± 1.5†††***	183.8 ± 4.9†††***	5.9 ± 0.17†††***	164.3 ± 6.1†††***	−7.31 ± 0.53†††***	−14.98 ± 1.03†††***
OVX + menatetrenone 3 mg/kg	20.2 ± 1.3**	88.5 ± 1.9	3.5 ± 0.08***	283.7 ± 9.0***	1.64 ± 0.09*	2.32 ± 0.46***
OVX + menatetrenone 30 mg/kg	23.6 ± 2.3	92.0 ± 4.7	3.8 ± 0.10†***	262.6 ± 7.4+**	1.42 ± 0.19	1.05 ± 0.65*

Each value represents mean ± SEM (n = 6–8)
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. Sham control group
†$P < 0.05$, †††$P < 0.001$ vs. OVX control group

Table 3. Coefficient of determination (R^2) values for the mechanical strength of lumbar vertebra by simple regression analysis

Group	The fifth lumbar vertebra					
		The microarchitectural indices				
	BMD	BV/TV	Tb.Th	Tb.N	TBPf	SMI
Sham-OVX groups	0.824**	0.784**	0.389**	0.800**	0.786**	0.641**
OVX-alfacalcidol treated groups a (0.1, 0.2 μg/kg)	0.921**	0.884**	0.744**	0.871**	0.891**	0.831**
OVX-menatetrenone treated groups (3, 30 mg/kg)	0.278*	0.176	0.054	0.382**	0.247	0.101

Rats were bilaterally ovariectomized at 10 months of age. Alfacalcidol was administered orally 5 times a week and menatetrenone was given as a dietary supplement for 6 months. *$P < 0.05$, **$P < 0.01$ (simple regression)

values of cross-sectional area were comparable to that in the OVX group. OVX caused an increase in the bone marrow area, and the difference was significant compared with that in the sham-operated group. In the ALF-treated groups, however, the values of marrow area decreased significantly compared with the OVX group and were smaller than that in the sham-operated group (Table 4). VK treatment also decreased the marrow area, but this effect was not dose dependent. ALF prevented the decrease in the cortical bone area caused by OVX and increased the cortical area to the level higher than that in the sham-operated group, whereas no significant effect of VK on the cortical area was observed (Table 4).

The Kinetic Parameters of Periosteal Perimeter. No significant changes in the MS/BS and the BFR/BS were observed in the OVX control group. These parameters were significantly higher in the ALF-treated groups than in the OVX group (Table 4). In the VK-treated groups, none of these parameters showed marked changes.

The Kinetic and Static Parameters of Endocortical Perimeter. The MS/BS and the BFR/BS, the parameters for bone formation, were significantly higher in the OVX group than in the sham-operated group. Although the increase in these parameters caused by OVX was suppressed by treatment with ALF at a dose of 0.1 μg/kg, these indices in the ALF 0.2 μg/kg-treated group were maintained at the level of the OVX group (Table 4). The N.Oc/BS and the ES/BS, the bone resorption parameters, in the OVX group were significantly higher than in the sham-operated group, but these parameters in the ALF-treated group decreased significantly compared with the OVX group (Table 4). VK treatment decreased both parameters for bone formation and resorption compared with the OVX group in a dose-dependent fashion, but the differences were not significant.

Effects of Six Months Treatment with Alfacalcidol and Menatetrenone on Material Strength of Femur (Fig. 4)

In order to investigate the effect of ALF and VK on the bone quality of the mid-femoral cortex, the material strength of femur was measured. Although the material strength in the OVX group was significantly lower than in the sham-operated group, ALF treatment restored the decrease of the material strength after OVX and maintained it at the same level as the sham-operated group (Fig. 4). The material strength was significantly higher in the VK 30 mg/kg-treated group than in the OVX group, which was also maintained at the sham level (Fig. 4).

Discussion

In this study, we clearly demonstrated that in the estrogen-deficient rat model of osteoporosis, ALF as well as VK prevented the bone loss and fragility of the spine and the femur. Our results also indicated that there were marked differences between ALF and VK in their efficacy and characteristics for improving the mechanical strength of the lumbar vertebra and the femoral diaphysis. Treatment with VK at a dose of 30 mg/kg required 6 months to exert a preventive effect on bone loss following estrogen deficiency, whereas ALF increased both the bone mass and the strength in 3 months and did not cause hypercalcemia, despite administration for as long as 6 months. ALF ameliorated the fragility in bone by a marked suppression of bone resorption while stimulating formation, and improved the bone strength with normal quality. Although VK had little effect on the bone turnover and the morphometric parameters, it might affect bone quality and thereby maintained the mechanical strength of normal level. Akiyama et al. [14] have reported the effects of VK on bone loss and fragility in OVX rats by using doses of 3–100 mg/kg per day, suggesting that the effective dose of VK in the OVX rat model is 3–30 mg/kg per day. In the present study, it was revealed that VK at a dose of 30 mg/kg required 6 months of treatment to prevent the bone loss caused by OVX.

Ovariectomized animals have been widely used as an experimental model of osteoporosis. This model is highly reproducible and characterized by loss of bone mass due to high bone turnover in which both bone resorption and formation are enhanced because of estrogen deficiency [30]. In this study, the serum ALP levels in OVX rats were higher than in sham-operated rats, which was consistent with the results of some studies by Akiyama et al. [14] and Kalu et al. [31], and OVX also caused a significant increase in urinary D-Pyr excretion as a bone resorption marker. Thus, it was confirmed that the OVX rat model showed high bone turnover after estrogen deficiency. The urinary D-Pyr excretion in the group treated with ALF for 3 months was reduced to the level in the sham-operated rats, suggesting that ALF acts on bone as a potent inhibitor of bone resorption, which was consistent with the results of the histomorphometric analysis at femoral diaphysis. In agreement with previous reports [14, 32], our results showed that the serum ALP level in VK 30 mg/kg-treated groups was lower than in OVX control groups. Although VK has been reported to be involved in the bone formation, our results indicated that assessment of the biochemical and the morphometric parameters failed to show any involvement of VK in the bone formation.

In recent years, 3-dimensional analysis of the human vertebrae has become possible using a micro-CT [33]. It has been reported that the treatment with fluorides increased the bone mineral content but failed to restore the

Table 4. Effects of alfacalcidol and menatetrenone on histomorphometric parameters of the midfemoral cortex in OVX rats (6-month treatment)

Group	Cross-sectional morphology			Histomorphometric parameters in periosteal perimeter	
	Total cross-sectional area (mm^2)	Bone marrow area (mm^2)	Cortical bone area (mm^2)	MS/BS (%)	BFR/BS ($\mu m^3/\mu m^2$/year)
Sham	9.1 ± 0.22	3.4 ± 0.13	5.7 ± 0.17	29.9 ± 8.64	51.0 ± 16.57
OVX + vehicle	9.7 ± 0.18	4.5 ± 0.08***	5.3 ± 0.13	30.0 ± 2.82	453 ± 5.74
OVX + alfacalcidol 0.1 μg/kg	9.7 ± 0.21	3.3 ± $0.07^{\dagger\dagger\dagger}$	6.3 ± $0.17^{\dagger\dagger\dagger}_{*}$	62.7 ± $5.96^{\dagger\dagger\dagger}_{**}$	107.1 ± $1231^{\dagger\dagger\dagger}_{*}$
OVX + alfacalcidol 0.2 μg/kg	9.9 ± 0.19*	2.9 ± $0.08^{\dagger\dagger\dagger}_{**}$	7.0 ± $0.18^{\dagger\dagger\dagger}_{***}$	63.4 ± $4.27^{\dagger\dagger\dagger}_{**}$	116.5 ± $8.94^{\dagger\dagger\dagger}_{**}$
OVX + menatetrenone 3 mg/kg	9.0 ± $0.14^{\dagger}$	3.8 ± $0.17^{\dagger\dagger}$	5.2 ± 0.10	27.2 ± 3.19	38.2 ± 5.27
OVX + menatetrenone 30 mg/kg	9.6 ± 0.21	4.2 ± 0.18^{*}	5.5 ± 0.13	33.9 ± 5.89	53.5 ± 10.62

Group	Histomorphometric parameters in endocortical perimeter			
	MS/BS (%)	BFR/BS ($\mu m^3/\mu m^2$/year)	N.Oc/BS (/mm)	ES/BS (%)
Sham	11.7 ± 1.99	23.8 ± 5.38	3.59 ± 0.26	27.1 ± 1.86
OVX + vehicle	20.3 ± 2.41*	56.8 ± 7.06**	5.23 ± 0.66*	44.0 ± 2.54***
OVX + alfacalcidol 0.1 μg/kg	15.7 ± 0.90	35.7 ± $4.04^{\dagger}$	1.50 ± $0.51^{\dagger\dagger\dagger}_{**}$	17.8 ± $3.80^{\dagger\dagger\dagger}_{*}$
OVX + alfacalcidol 0.2 μg/kg	19.7 ± 1.70**	48.2 ± 5.59**	0.53 ± $0.13^{\dagger\dagger\dagger}_{***}$	8.9 ± $1.21^{\dagger\dagger\dagger}_{***}$
OVX + menatetrenone 3 mg/kg	20.4 ± 2.16**	51.4 ± 6.78**	4.03 ± 0.49	40.6 ± 2.28***
OVX + menatetrenone 30 mg/kg	15.5 ± 1.37	37.9 ± 3.20	3.37 ± 0.53	35.8 ± 2.71*

Each value represents the mean ± SEM (n = 6–8)
$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. Sham control group
$^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ vs. OVX control group

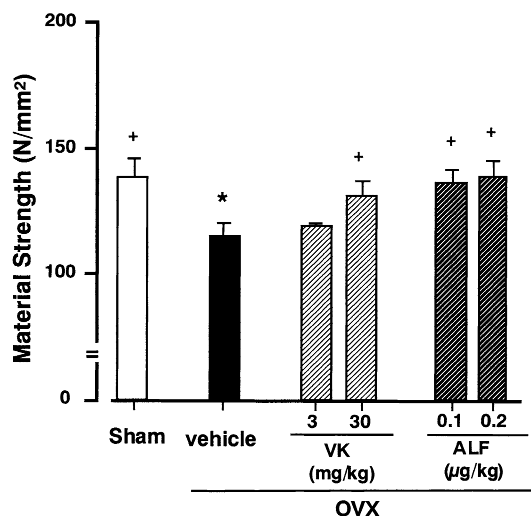

Fig. 4. Material strength of femoral midshaft in OVX rats treated with alfacalcidol or menatetrenone for 6 months. Each value represents the mean + SEM (n = 6–8). $^{*}P < 0.05$ compared with the sham-operated group. $^{+}P < 0.05$ compared with the OVX-control group.

mechanical strength of the vertebra [34, 35], suggesting that the mechanical property of bone depends not only on bone mass but also on the quality and the structure of bone. Clinical and preclinical studies have reported that the trabecular structure is a critical factor for the strength of lumbar vertebra [4, 36]. In the present study, 3D images of the lumbar vertebra obtained by micro-CT analysis showed that OVX caused the trabecular bone to become thin and sparse in cancellous bone. It was also revealed that (1) ALF increased the interconnections in trabecular bone and converted the rod structures into the plate structures; (2) in the ALF-treated groups there was a high correlation between the structural parameters and the mechanical strength, suggesting that an increase in spinal strength by ALF administration is closely associated not only with an increase in the BMD but also ameliorating the bone structure. In contrast, a 3D visual assessment of the rendered images showed that VK treatment prevented the deterioration of trabecular bone up to almost the same level as in the sham-operated group, which was consistent with the previous report on the proximal region of rat tibia [32], but nevertheless the correlation between each of the structural indices and the spinal strength was low in the VK-treated groups. It indicated that some factors, including bone quality, other than the trabecular microstructure might be important determinants of the increase in the spinal strength by VK administration.

To determine the mechanisms of these drugs in improving the mechanical strength of the femur, the kinetic and static parameters at the femoral diaphysis were assessed. Our results of the histomorphometric analysis demonstrated that ALF caused a significant suppression of bone resorption and yet maintained formation in endocortical perimeter, while stimulating bone formation at the periosteal perimeter, resulting in an increase of the cortical bone area. These results are consistent with previous studies on the effects of active vitamin D_3 *in vivo* [7, 8, 37–39]. No apparent effects of VK on these histomorphometric parameters were observed, whereas VK treatment maintained the material strength in the femoral diaphysis of the normal level, as also did ALF, suggesting that VK affected bone quality of mid-femoral cortex and thereby prevented the decrease of femoral strength by ovariectomy.

Clinical studies have reported that ALF as well as VK successfully inhibited the occurrence of new bone fractures, but LBMD values did not significantly increase in osteoporotic patients [5, 6, 10]. In this preclinical study using a rat model of high-turnover condition, ALF treatment resulted in a pronounced increase in BMD as well as bone strength. It is known that the remodeling pattern and calcium homeostasis in rats are not necessarily the same as those of humans. It is also understood that the remodeling period in rats is shorter than the period in humans. Thus, the marked increase in LBMD in rat model could be, at least in part, due to the larger number of remodeling cycles during the experimental period. Another explanation is a genetic difference in the sensitivity to vitamin D, as observed among the different vitamin D-receptor gene polymorphisms in humans [40].

In conclusion, it was demonstrated that the two drugs, ALF and VK, differed markedly in their potency for improving the strength of the spine as well as the femur, in the estrogen-deficient rat model of osteoporosis. VK had little effect on the bone turnover and the morphometric parameters, whereas it could affect the bone quality and restore the decrease of the mechanical strength by estrogen deficiency. In contrast, ALF could ameliorate the fragility in bone structure and improve the bone strength with normal quality more potently than VK by a marked suppression of bone resorption while stimulating formation. These results provide important clues in understanding the action mechanisms of these agents on bone metabolism in the treatment of postmenopausal osteoporosis.

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