Table 1. Changes in body weight

	Body weight (g)		
	Baseline (at surgery)	6 weeks (start of administration)	12 weeks
Sham	366 ± 32	381 ± 48	351 ± 38
OVX			
Vehicle	364 ± 29	446 ± 23[a]	404 ± 9[a]
RIS (0.1 mg/kg)	368 ± 27	440 ± 33[a]	411 ± 28[a]
RIS (1.0 mg/kg)	364 ± 27	431 ± 26[a]	403 ± 35[a]
RIS (2.5 mg/kg)	374 ± 40	440 ± 33[a]	404 ± 33[a]
ALF (0.5 μg/kg)	371 ± 30	436 ± 24[a]	388 ± 30[a]

Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. [a]: significant vs Sham.

Table 2. Biochemical markers

	Deoxypyridinoline (nmol/mmol Cr)			Osteocalcin (ng/ml)	Calcium (mg/dl)	Phosphorus (mg/dl)
	6 weeks (start of administration)	9 weeks	12 weeks			
Sham	19.6 ± 7.4	17.0 ± 3.7	14.8 ± 5.1	21.8 ± 5.3	9.7 ± 0.2	6.4 ± 0.3
OVX						
Vehicle	44.3 ± 10.9[a]	58.3 ± 18.4[a]	54.5 ± 20.6[a]	33.3 ± 5.8[a]	9.9 ± 0.3	6.7 ± 0.3
RIS (0.1 mg/kg)	49.7 ± 18.3[a]	42.6 ± 8.5[ab]	29.3 ± 5.6[ab]	30.8 ± 7.3[a]	9.6 ± 0.5	5.8 ± 0.4[ab]
RIS (1.0 mg/kg)	50.3 ± 14.7[a]	22.3 ± 5.9[bc]	16.4 ± 4.4[bc]	23.8 ± 8.1[b]	9.5 ± 0.4	5.6 ± 0.5[ab]
RIS (2.5 mg/kg)	48.7 ± 14.8[a]	25.7 ± 9.9[bc]	13.9 ± 3.8[bc]	13.9 ± 3.0[bcd]	9.4 ± 0.2[b]	5.1 ± 0.2[abcd]
ALF (0.5 μg/kg)	49.8 ± 13.2[a]	38.7 ± 16.9[abde]	24.8 ± 10.7[be]	23.7 ± 12.1[be]	10.1 ± 0.6[f]	6.9 ± 0.6[ace]

Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. [a]: significant vs Sham, [b]: significant vs Vehicle, [c]: significant vs RIS (0.1 mg/kg), [d]: significant vs RIS (1.0 mg/kg), [e]: significant vs RIS (2.5 mg/kg), [f]: significant vs RIS (all doses).

relative to the values observed in the OVX-Vehicle group, but the values were not restored to those observed in the Sham group. The effects of RIS (1.0 mg/kg and 2.5 mg/kg) were greater than those of RIS (0.1 mg/kg). The OVX-induced decrease of the Tb Th was entirely prevented by RIS (at all the doses), with the value of this parameter being restored to the value observed in the Sham group. ALF also increased the BV/TV and Tb N and decreased the Tb Sp relative to the values observed in the OVX-Vehicle group, but the values were not restored to those observed in the Sham group. Furthermore, ALF also increased the Tb Th and the increase of this parameter following ALF treatment was more marked than that following RIS treatment (at all the doses). Thus, the alterations of the structural parameters induced by RIS (at all the doses) were attributable to suppression of the increase of bone resorption (ES/BS) and formation (BFR/BS, BFR/BV), and the alterations of the structural parameters induced by ALF were attributable to suppression of the increase of bone resorption (ES/BS), while bone formation (ObS/BS, MS/BS, BFR/BS) was maintained. The effect of ALF on cancellous BV/TV was similar to that of RIS (1.0 mg/kg and 2.5 mg/kg).

Bone histomorphometric analysis of the cortical bone of the tibial diaphysis (Fig. 2 and Table 4)

OVX did not affect the Tt At or Ct Ar, despite stimulated periosteal bone formation (MS/BS, BFR/BS), but increased the Ma Ar as a result of increased endocortical bone resorption (ES/BS) and, subsequently, increased endocortical bone formation (BFR/BS). RIS (at all the doses) prevented the increase of the Ma Ar, restoring it to the value observed in the Sham group. On the other

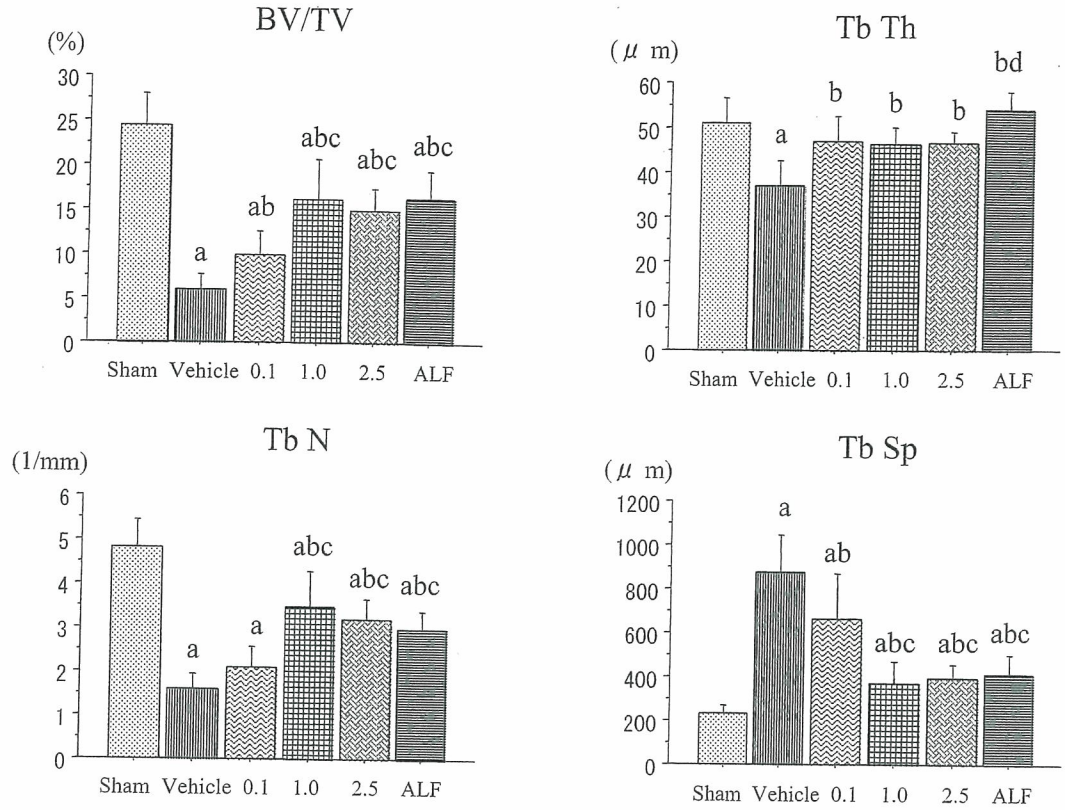

Fig. 1. Bone histomorphometric analysis of the cancellous bone of the proximal tibial metaphysis. —Structural parameters—. Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. Sham: Sham-operated control, Vehicle: OVX+Vehicle, 0.1: OVX+RIS (0.1 mg/kg), 1.0: OVX+RIS (1.0 mg/kg), 2.5: OVX+RIS (2.5 mg/kg), ALF: OVX+ALF. a: significant vs Sham, b: significant vs Vehicle, c: significant vs RIS (0.1 mg/kg), d: significant vs RIS (all doses). BV/TV: bone volume/total tissue volume, Tb N: trabecular number, Tb Th: trabecular thickness, Tb Sp: trabecular separation.

Table 3. Histomorphometric analysis of the cancellous bone of the proximal tibial metaphysis —Formative and resorptive variables—

	ES/BS (%)	N.Oc/BS (#/mm)	OcS/BS (%)	ObS/BS (%)	MS/BS (%)	MAR (μm/day)	BFR/BS (μm³/μm²/day)	BFR/BV (%/year)
Sham	7.2 ± 3.4	1.11 ± 0.20	4.2 ± 0.6	13.4 ± 0.5	10.6 ± 4.5	0.96 ± 0.17	9.9 ± 4.3	119 ± 50
OVX								
Vehicle	12.6 ± 4.2[a]	4.40 ± 1.00[a]	15.4 ± 2.5[a]	19.6 ± 2.5[a]	19.2 ± 3.6[a]	0.88 ± 0.12	17.2 ± 5.7[a]	281 ± 63[a]
RIS (0.1 mg/kg)	8.0 ± 2.0[b]	1.82 ± 0.35[ab]	6.6 ± 1.8[ab]	15.3 ± 2.9[b]	15.7 ± 4.2[a]	0.77 ± 0.19	12.1 ± 4.4[b]	156 ± 53[b]
RIS (1.0 mg/kg)	7.5 ± 2.4[b]	1.70 ± 0.39[ab]	5.9 ± 1.3[ab]	12.0 ± 2.7[bc]	13.5 ± 3.4[b]	0.67 ± 0.19[ab]	8.9 ± 2.8[b]	117 ± 33[b]
RIS (2.5 mg/kg)	6.2 ± 1.5[b]	1.76 ± 0.51[ab]	6.3 ± 1.7[ab]	13.0 ± 2.4[b]	10.5 ± 3.8[bc]	0.56 ± 0.07[abc]	5.9 ± 2.4[bc]	76 ± 28[bc]
ALF (0.5 μg/kg)	7.6 ± 3.1[b]	2.14 ± 0.45[ab]	7.9 ± 1.8[ab]	18.8 ± 2.1[af]	22.4 ± 4.1[af]	0.86 ± 0.26[de]	19.1 ± 5.6[af]	213 ± 53[abf]

Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. [a]: significant vs Sham, [b]: significant vs Vehicle, [c]: significant vs RIS (0.1 mg/kg), [d]: significant vs RIS (1.0 mg/kg), [e]: significant vs RIS (2.5 mg/kg), [f]: significant vs RIS (all doses). ES: eroded surface, BS: bone surface, N.Oc: number of osteoclast, ObS: osteoblast surface, MS: mineralizing surface, MAR: mineral apposition rate, BFR: bone formation rate, BV: bone volume.

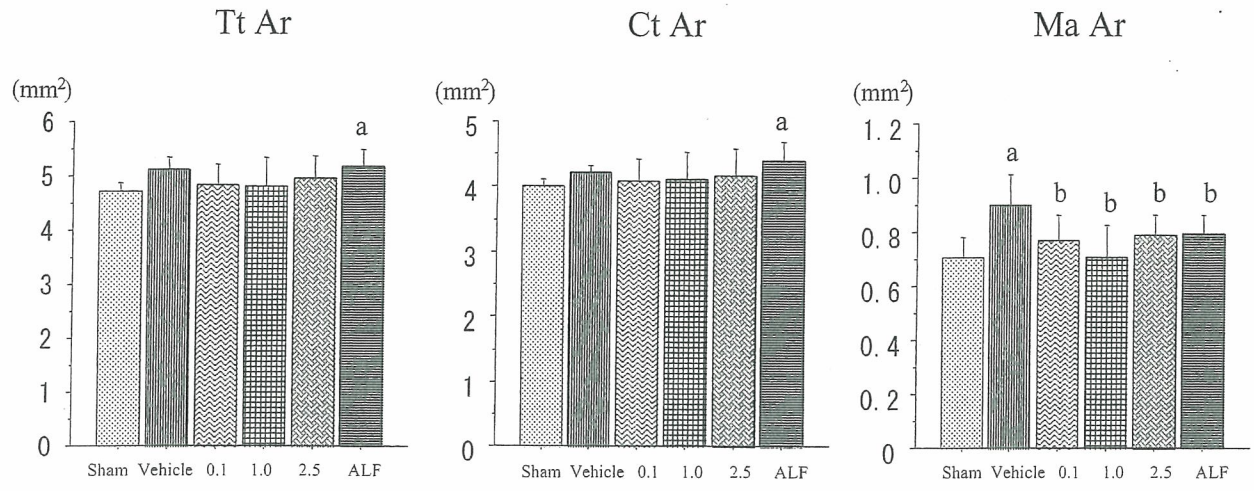

Fig. 2. Bone histomorphometric analysis of the cortical bone of the tibial diaphysis. —Structural parameters—. Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. Sham: Sham-operated control, Vehicle: OVX+Vehicle, 0.1: OVX+RIS (0.1 mg/kg), 1.0: OVX+RIS (1.0 mg/kg), 2.5: OVX+RIS (2.5 mg/kg), ALF: OVX+ALF. a: significant vs Sham, b: significant vs Vehicle. Tt Ar: total tissue area, Ct Ar: cortical area, Ma Ar: marrow area.

Table 4. Histomorphometric analysis of the cortical bone of the tibial diaphysis —Formative and resorptive variables—

	Periosteal			Endocortical			
	MS/BS (%)	MAR (μm/day)	BFR/BS (μm³/μm²/day)	ES/BS (%)	MS/BS (%)	MAR (μm/day)	BFR/BS (μm³/μm²/day)
Sham	39.6 ± 10.7	1.49 ± 0.22	59.7 ± 19.7	27.7 ± 3.5	5.5 ± 1.2	0.00 ± 0.00	0.0 ± 0.0
OVX							
Vehicle	66.4 ± 10.2[a]	1.51 ± 0.24	99.8 ± 17.3[a]	40.0 ± 5.0[a]	12.9 ± 5.9[a]	0.63 ± 0.63[a]	9.8 ± 12.3[a]
RIS (0.1 mg/kg)	60.9 ± 13.3[a]	1.26 ± 0.25	77.2 ± 22.7	33.7 ± 5.4	13.3 ± 4.7[a]	0.15 ± 0.28[b]	2.5 ± 4.7
RIS (1.0 mg/kg)	59.1 ± 10.6[a]	1.19 ± 0.27[ab]	70.8 ± 23.2	33.9 ± 7.0	8.1 ± 3.8[c]	0.16 ± 0.30[b]	1.9 ± 3.6
RIS (2.5 mg/kg)	57.1 ± 23.2	1.17 ± 0.26[b]	69.2 ± 36.4	26.6 ± 10.0[b]	6.0 ± 4.6[bc]	0.13 ± 0.35[b]	1.2 ± 3.4[b]
ALF (0.5μg/kg)	53.8 ± 20.0	1.69 ± 0.22[f]	92.9 ± 43.8	25.6 ± 11.0[bd]	18.3 ± 6.5[ae]	1.29 ± 0.23[abe]	24.1 ± 10.6[abe]

Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. [a]: significant vs Sham, [b]: significant vs Vehicle, [c]: significant vs RIS (0.1 mg/kg), [d]: significant vs RIS (1.0 mg/kg), [e]: significant vs RIS (2.5 mg/kg), [f]: significant vs RIS (all doses). MS: mineralizing surface, BS: bone surface, MAR: mineral apposition rate, BFR: bone formation rate, ES: eroded surface.

hand, only RIS (2.5 mg/kg) suppressed endocortical bone resorption (ES/BS) and formation (MS/BS, MAR, BFR/BS). ALF increased the Tt Ar and Ct Ar to beyond the values observed in the Sham group, and prevented the increase of the Ma Ar after OVX. Furthermore, it also suppressed endocortical bone resorption (ES/BS), while even stimulating endocortical bone formation (MS/BS, BFR/BS) to beyond the values observed in the OVX-Vehicle group.

Biomechanical test of the femur (Table 5)

OVX decreased the maximum load of the femoral distal metaphysis, without any effect on the mechanical properties of the femoral diaphysis. RIS (at all the doses) had no effects on the mechanical properties of the femoral distal metaphysis or diaphysis, whereas ALF prevented the loss of the maximum load and increased the breaking energy of the femoral distal metaphysis, without any effect on the mechanical properties of the femoral diaphysis.

Table 5. Mechanical properties of the femur

	Distal metaphysis			Diaphysis		
	Maximum load (N)	Stiffness (N/cm)	Breaking energy ($x10^{-5}N \cdot m$)	Maximum load (N)	Stiffness (N/cm)	Breaking energy ($x10^{-5}N \cdot m$)
Sham	362 ± 67	526 ± 142	45885 ± 11076	120 ± 6	167 ± 24	4956 ± 917
OVX						
Vehicle	279 ± 28[a]	388 ± 106	41079 ± 3598	131 ± 19	169 ± 44	6175 ± 1746
RIS (0.1 mg/kg)	313 ± 27	436 ± 72	44135 ± 10637	132 ± 9	174 ± 21	6262 ± 937
RIS (1.0 mg/kg)	312 ± 35	464 ± 52	49013 ± 5927	132 ± 14	179 ± 35	6591 ± 1575
RIS (2.5 mg/kg)	335 ± 39	502 ± 132	47561 ± 6154	132 ± 18	170 ± 42	6976 ± 2839
ALF (0.5 μg/kg)	404 ± 76[bc]	551 ± 177[b]	58193 ± 10422[ac]	131 ± 9	170 ± 16	6904 ± 1397

Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. [a]: significant vs Sham, [b]: significant vs Vehicle, [c]: significant vs RIS (all doses).

Discussion

The present study showed the differential effects of RIS and ALF on cancellous and cortical bone in ovariectomized osteopenic rats. The strengths of this study are the detailed bone histomorphometric analyses of cancellous and cortical bone, the measurement of biochemical markers of bone turnover, and the measurement of the mechanical properties of the femoral distal metaphysis. The weaknesses are the ineffectiveness of both treatments in restoring the cancellous BV/TV to the osteopenic skeleton after OVX, despite decreased multiple parameters related to bone remodeling. The two treatments increased the cancellous BV/TV compared with OVX-Vehicle-controls, but could not restore to the values seen in Sham-controls. Because the two treatments were impotent, switching to potent anabolic agents such as parathyroid hormone (PTH) might completely restore the cancellous BV/TV in ovariectomized osteopenic rats.

The effects of OVX on cancellous and cortical bone have already been established, and our results can be comparative with those of a number of previous studies. In particular, we confirmed that OVX resulted in cancellous osteopenia by 6 weeks after surgery in 6-month-old rats, without inducing cortical osteopenia because of the absence of Haversian-based remodeling in rat cortical bone [11]. According to this report, our study animals would also have developed cancellous osteopenia by 6 weeks after the OVX.

Bisphosphonates inhibit osteoclast-mediated bone resorption, and loss of osteoclast function and apoptosis is a consequence of loss of function of one or more important signaling proteins. A nitrogen-containing bisphosphonate like RIS is not metabolized but can inhibit enzymes of the mevalonate pathway, thereby preventing the biosynthesis of isoprenoid compounds that are essential for the post-translational modification of small GTPases [18]. RIS has a potent anti-resorptive effect on the bone.

RIS improved not only the connectivity of trabecular bone, but also its thickness by suppressing bone turnover in ovariectomized osteopenic rats. The effects of RIS (1.0 mg/kg) and RIS (2.5 mg/kg), which were similar, were more marked than those of RIS (0.1 mg/kg). Therefore, 1.0 mg/kg was considered to be the minimum effective dose of RIS in the present study. However, RIS did not completely restore the cancellous BV/TV to the value observed in the Sham group, reflecting its limitation in increasing cancellous bone mass. It is possible that RIS reduced the amount of remodeling space and then increased the cancellous bone mass. The decreases of the serum calcium and phosphorus levels after RIS treatment, with the greatest decreases by RIS (2.5 mg/kg), suggest that RIS treatment resulted in accumulation of minerals in the bone. The effect of RIS on bone metabolism was similar in ovariectomized rats with and without established osteopenia [6, 15].

The active and hormonal form of vitamin D, 1α, 25-dihydroxyvitamin D_3, plays a central role in bone and mineral homeostasis through binding to the vitamin D receptor in calcium-related target organs, including the intestine, bone, kidney, and parathyroid gland [1, 17].

One α, $25(OH)_2D_3$ stimulates calcium absorption from the intestine, regulates bone resorption as well as formation, and enhances calcium reabsorption in the distal renal tubules, while it represses parathyroid hormone gene transcription in the parathyroid glands [1, 17]. One α-hydroxyvitamin D_3 (ALF), which is the prodrug of 1α, 25-dihydroxyvitamin D_3, has been widely used in the treatment of a variety of metabolic bone diseases, such as rickets/osteomalacia, renal osteodystrophy, and osteoporosis [1, 17]. A clinical study showed that ALF reduced bone turnover and prevented vertebral fractures in postmenopausal women with osteoporosis [14]. However, ALF shows a relatively low effectiveness and the risk of developing hypercalciuria/hypercalcemia and urinary stones, resulting in a relatively narrow therapeutic window [4]. An experimental study, which was conducted to investigate the preventive effect of ALF on osteopenia in ovariectomized rats, clearly showed that ALF suppressed bone resorption, but maintained or even stimulated bone formation [20].

ALF improved not only the connectivity of trabecular bone, but also its thickness by suppressing bone turnover but maintaining bone formation in ovariectomized osteopenic rats. These alterations in bone formation and resorption in ovariectomized osteopenic rats were similar to those observed in a previous study that examined the preventive effect of ALF on the cancellous bone loss in ovariectomized rats [20]. The effect of ALF on the cancellous BV/TV was similar to that of RIS (1.0 mg/kg and 2.5 mg/kg). However, the suppression of bone turnover by ALF was milder than that by RIS (at all the doses). Thus, ALF had a milder antiresorptive effect than RIS on cancellous bone in ovariectomized osteopenic rats, and also appeared to have the potential to maintain bone formation, differing in this respect from RIS. The increase in the Tb Th induced by ALF was more marked than that induced by RIS (at all the doses), probably due to maintained bone formation. However, the cancellous BV/TV was not restored to the level observed in the Sham group, reflecting the limitation of ALF at our dose setting in increasing the cancellous bone mass.

OVX decreased the maximum load of the femoral distal metaphysis, associated with a decrease in the cancellous BV/TV. Despite the similar effects of RIS and ALF on the cancellous BV/TV, RIS (at all the doses) had no effects on the mechanical properties of the femoral distal metaphysis, whereas ALF prevented the loss of the maximum load and increased the breaking energy of the femoral distal metaphysis. These results may partly be attributable to the more pronounced effect of ALF than RIS on the Tb Th. The Tb Th may be an important factor in determining the bone strength, as observed in rats treated with vitamin K_2 [9, 10]. Thus, the efficacy of ALF in improving the mechanical properties of the bone in ovariectomized osteopenic rats observed in the present study might be attributable, at least in part, to the marked increase of the Tb Th induced by the drug.

Clinically, it is apparent that RIS is more effective than ALF in increasing the lumbar BMD and reducing the incidence of vertebral fractures in postmenopausal osteoporotic women [3]. Nevertheless, in the present study, ALF induced an increase of the cancellous BV/TV similar to that of RIS. This discrepancy between the clinical and experimental results may be due to the differential responses of cancellous bone to ALF between ovariectomized osteopenic rats and postmenopausal osteoporotic women (humans). In fact, the potent preventive effect of alfacalcidol on cancellous bone loss after OVX in rats has been confirmed in the previous studies [20]; thus, ALF might exert greater beneficial effects on rat bones than on human bones.

Increased endocortical bone resorption and periosteal bone formation seem to be similar in ovariectomized osteopenic rats and postmenopausal osteoporotic women [19]. Thus, the pharmacological effects of the drugs on the endocortical bone in ovariectomized osteopenic rats can be exactly translatable into clinically beneficial effects on the endocortical bone in postmenopausal osteoporotic women. ALF, but not RIS, increased the Ct Ar, mainly by decreasing endocortical bone resorption and increasing endocortical bone formation. However, perhaps because this effect was modest, the mechanical properties of the femoral diaphysis were not improved. Also, RIS did not decrease endocortical bone resorption in the present study, probably because osteoclasts on the endocortical surface may be less responsive to bisphosphonates than those on the trabecular surface [2].

In conclusion, the present study demonstrated that RIS and ALF increased the cancellous bone mass by suppressing bone turnover in ovariectomized osteopenic

rats. The effects of both ALF and RIS (at effective doses) were similar on the cancellous bone mass. ALF was associated with maintained or even stimulated bone formation and a marked increase of the trabecular thickness. Also, only ALF increased the cortical bone mass, and prevented the loss of the maximum load of the metaphysis of the femur. Thus, the present study showed the differential effects of RIS and ALF on cancellous and cortical bone in ovariectomized osteopenic rats.

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Preventive Effects of Risedronate and Calcitriol on Cancellous Osteopenia in Rats Treated with High-Dose Glucocorticoid

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Abstract: *We compared the effects of risedronate (Ris) and calcitriol (Cal) on cancellous osteopenia in rats treated with high-dose glucocorticoid (GC). Forty female Sprague-Dawley rats, 4 months of age, were randomized by the stratified weight method into four groups of 10 rats each according to the following treatment schedule: intact control, and GC administration with vehicle, Ris, or Cal. The GC (methylprednisolone sodium succinate, 5.0 mg/kg, s.c.), Ris (10 μg/kg, s.c.), and Cal (0.1 μg/kg, p.o.) were administered 3 times a week. At the end of the 4-week treatment period, bone histomorphometric analysis was performed for cancellous bone of the proximal tibial metaphysis. The GC administration decreased cancellous bone volume (BV/total tissue volume [TV]), trabecular number (Tb N), and trabecular thickness (Tb Th), as a result of increased bone resorption and decreased bone formation. Ris treatment markedly increased cancellous BV/TV and Tb N above the control level as a result of suppressed bone turnover. On the other hand, Cal treatment attenuated the GC-induced decrease in cancellous BV/TV and Tb Th as a result of suppressed bone resorption and maintained bone formation. This study showed the differential effects of Ris and Cal on cancellous osteopenia in rats treated with high-dose GC.*

Key words: *calcitriol, cancellous osteopenia, glucocorticoid, rat, risedronate*

Introduction

Glucocorticoid (GC) therapy has been associated with an increased risk of osteoporosis, and consequently, an increased incidence of fractures. The loss of bone mineral density (BMD) is more evident in cancellous bone than in cortical bone [23]. Several meta-analyses/systematic-reviews have reported the effects of vitamin D,

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calcitonin, and bisphosphonates on the BMD of the lumbar spine and hip in patients treated with GCs; vitamin D (both active and native vitamin D) and calcitonin stabilize lumbar spine BMD, while bisphosphonates stabilize both lumbar spine and hip BMD, with, nonetheless, a less certain effect on hip BMD [2, 8, 9, 21]. In particular, bisphosphonates have been reported to be the most effective in stabilizing lumbar BMD in patients treated with GCs [1]. In Japan, it is generally agreed that bisphosphonates should be the first line drugs in the treatment of GC-induced osteoporosis, while active vitamin D is the second line treatment [14]. However, the countermeasures against the rapid and vigorous loss of BMD by high-dose GC therapy [24], i.e., pulse steroid therapy are less certain, and the mechanism for the efficacy of bisphosphonates and active vitamin D in stabilizing lumbar BMD in patients treated with GCs in terms of the effect of these drugs on cancellous microarchitecture remains to be established. The purpose of the present study was to use an animal model to compare the effects of risedronate (Ris) and calcitriol (Cal) on high-dose GC-induced cancellous osteopenia by means of bone histomorphometry.

Materials and Methods

Treatment of animals

Forty female Sprague-Dawley rats, 4 months of age, were purchased from Hilltop Lab. Animals, Inc. (Scottdale, PA, USA). The animals were housed under local vivarium conditions (temperature 23.8°C and 12 h on/off light cycle), and were fed a pelleted standard chow diet containing 1.36% calcium and 2,400 IU/kg vitamin D (Rodent Diet 8604, Harlan Teklad, Madison, WI, USA), with free access to water. After allowing one-week's adaptation to the new environment, the rats were randomized by the stratified weight method into four groups of 10 rats each according to the following treatment schedule: intact control (CON), and GC administration with vehicle, Ris, or Cal as preventive treatment. Methylprednisolone sodium succinate (Pharmacia & Upjohn Company, Kalamazoo, MI, USA) was administered as the GC, at a dose of 5.0 mg/kg body weight, three times a week by subcutaneous injection. Ris (Aventis Pharma, Tokyo, Japan) was dissolved in 0.1 ml of sterile saline, and then administered by subcutaneous injection at a dose of 10 μg/kg body weight three times a week. Cal (Chugai, Tokyo, Japan) was dissolved in 0.1 ml of PBS containing 0.25% ethanol and 0.1% Tween 20, and then administered by gavage deep into the mouth at a dose of 0.1 μg/kg body weight three times a week. The Ris and Cal doses were considered to be effective, in accordance with previously published data [10, 11, 13]. The body weight of the rats was monitored weekly and the experimental period was 4 weeks. The study was carried out at Winthrop-University Hospital, and the animals were maintained according to the National Institutes of Health (NIH) Guidelines for Care and Use of Laboratory Animals. All the animal protocols were approved by the Laboratory Animal Care Committee of Winthrop-University Hospital.

Preparation of specimens

All the rats were labeled with 10 mg/kg of calcein (Sigma Chemical, St. Louis, MO, USA) injected intramuscularly 10 days and 3 days before they were sacrificed. The animals were anesthetized with ketamine injected intraperitoneally at 80 mg/kg, together with xylazine at 12 mg/kg, and sacrificed by exsanguination. A serum specimen, the left femur and right tibia were collected from every animal.

The serum samples were stored at –20°C until use for the measurements of serum calcium and phosphorus levels with an automated instrument (Dada Behring Model RXL, Bakersfield, CA, USA). The femurs were stored at –20°C until use for BMD measurement as described below. The tibial length was measured with dial calipers and the bones were then used for bone histomorphometric analysis; they were fixed overnight in 40% cold ethanol, and then cut into three parts using an Isomet saw (Buehler, Lake Bluff, IL, USA). The proximal tibial metaphyses were stained with Villanueva Osteochrome Bone Stain (Polyscience, Warrington, PA, USA) for 5 days. The specimens were then dehydrated sequentially in ascending concentrations of ethanol (70%, 95%, and 100%) and xylene and then embedded in methyl-methacrylate (EM Science, Gibbstown, NJ, USA) at 4°C, in accordance with the method of Erben [3]. Frontal sections of the proximal tibial metaphysis were cut at 5 μm thickness using a microtome (Leica RM2155; Leica Inc., Nussloch, Germany), transferred onto chromium-gelatin-coated slides, dried overnight under pressure at 42°C, and coverslipped with Eukitt

mounting medium (Calibrated Instruments, Hawthorne, NY, USA) for static and dynamic histomorphometric analyses.

Femoral BMD

The BMD of the whole left femur was determined by dual energy X-ray absorptiometry (DXA) using a Hologic QDR-2000 plus (Hologic Inc., Bedford, MA, USA). The instrument was adapted for an ultra-resolution mode, with line spacing of 0.0254 cm, resolution of 0.0127 cm, and collimation of 0.9 cm diameter. The bone was placed in a Petri dish, and to simulate soft-tissue density, tap water was poured around the bone to a depth of 1 cm. The bone mineral content and bone area were measured, and the BMD of that area was calculated by dividing bone mineral content by bone area. The coefficient of variation of these measurements at our laboratory was less than 1.0% [20].

Bone histomorphometric analysis of the tibia

A digitizing morphometric system was used to measure bone histomorphometric parameters. The system consisted of an epifluorescence microscope (Nikon E-400, OsteoMetrics, Atlanta, GA, USA), an Osteomeasure High Resolution Color Subsystem (OsteoMetrics, Atlanta, GA, USA) coupled to an IBM computer, and a morphometry program (OsteoMetrics, Atlanta, GA, USA). The measured parameters for cancellous bone included total tissue volume (TV), bone volume (BV), bone surface (BS), eroded surface (ES), single- and double-labeled surfaces (sLS and dLS, respectively), and interlabel width. These data were used to calculate percent cancellous bone volume (BV/TV), trabecular number (Tb N), trabecular thickness (Tb Th), trabecular separation (Tb Sp), ES/BS, mineralizing surface (MS)/BS [(sLS/2+dLS)/BS], mineral apposition rate (MAR), bone formation rate (BFR)/BS, and BFR/BV, in accordance with the standard nomenclature proposed by Parfitt *et al.* [19]. In the present study, the region of cancellous bone measured was 1–4 mm distal to the lower margin of the growth plate in the proximal tibial metaphysis, which consists of secondary spongiosa. In addition to measurement of the above parameters, interlabel width beneath the growth plate was used to calculate longitudinal growth rate (LGR).

Statistical analysis

All the data were expressed as means and standard deviation (SD). Multiple comparisons of data among the groups were performed by analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) test. All statistical analyses were performed using the Stat View J-5.0 program on a Macintosh computer. A significance level of $P<0.05$ was used for all the comparisons.

Results

Body weight, tibial length, femoral BMD, and serum calcium and phosphorus levels (Table 1)

The initial body weight did not differ significantly among the groups.

The GC administration decreased body weight, tibial length, and serum calcium levels, but had no effect on femoral BMD.

Ris prevented the GC-induced decrease in tibial

Table 1. Body weight, tibial length, femoral BMD, and serum calcium and phosphorus levels

	Initial body weight (g)	Final body weight (g)	Tibial length (mm)	Femoral BMD (mg/cm^2)	Serum Calcium (mg/dl)	Serum Phosphorus (mg/dl)
CON	243 ± 6	293 ± 10	39.0 ± 0.5	223 ± 7	9.7 ± 0.2	8.0 ± 0.6
GC						
Vehicle	237 ± 10	272 ± 16[a]	38.2 ± 0.6[a]	219 ± 7	9.4 ± 0.1[a]	7.3 ± 0.6[a]
Ris	234 ± 9	271 ± 16[a]	38.9 ± 0.4[b]	225 ± 8	9.2 ± 0.2[ab]	7.3 ± 0.6[a]
Cal	243 ± 10	285 ± 13[bc]	38.7 ± 0.6[b]	226 ± 5	9.4 ± 0.3[ac]	7.1 ± 0.8[a]

Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. CON: intact control; GC: glucocorticoid; Ris: risedronate; Cal: calcitriol. [a], significant vs CON; [b], significant vs GC; [c], significant vs Ris. BMD: bone mineral density.

length, but showed no effect on femoral BMD; Ris also accelerated the GC-induced decrease in serum calcium levels. On the other hand, Cal prevented the GC-induced decrease in body weight and tibial length, but showed no effect on serum calcium levels, or femoral BMD.

Bone histomorphometric analysis of cancellous bone of the proximal tibial metaphysis (Figs. 1 and 2)

The GC administration decreased cancellous BV/TV, Tb N, and Tb Th, and increased Tb Sp. This cancellous osteopenia was associated with decreased bone formation (MS/BS, MAR, BFR/BS, BFR/BV), and increased ES/BS. The GC administration also decreased LGR.

Ris increased cancellous BV/TV and Tb N above the values observed in the CON group, and decreased Tb Sp to below the value observed in the CON group, as a result of markedly decreased bone turnover, indicated by decreases in ES/BS, BFR/BS and BFR/BV, while Cal attenuated the GC-induced decrease in cancellous BV/TV and Tb Th by decreasing ES/BS and maintaining MS/BS. Ris prevented the GC-induced decrease in LGR, whereas Cal had no effect on this parameter.

Discussion

GC induces cancellous osteopenia in rats. It has been demonstrated that GC induces the loss of cancellous BV/TV, Tb N, and Tb Th, decreases longitudinal

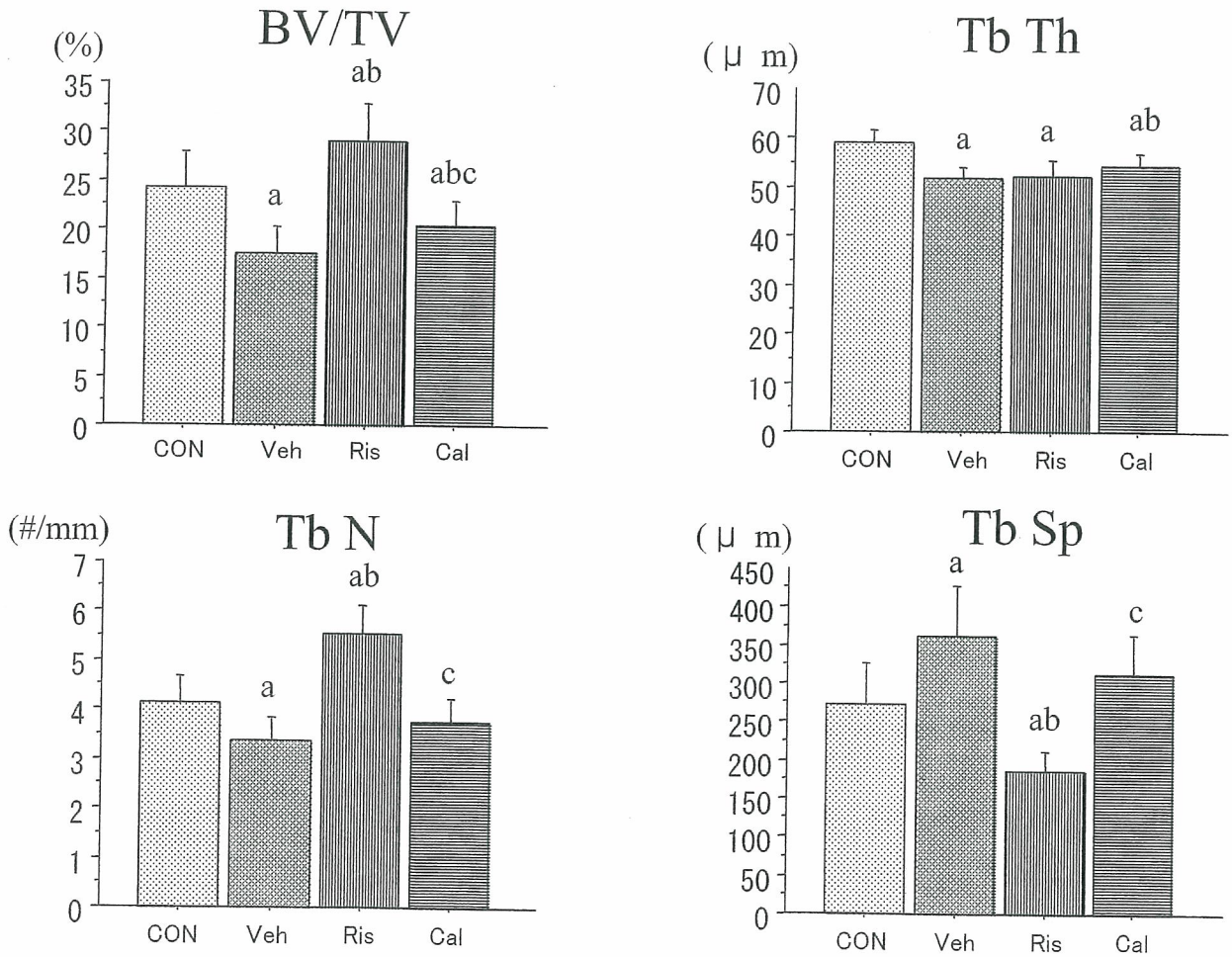

Fig. 1. Bone histomorphometric analysis of cancellous bone of the proximal tibial metaphysis. –Structural variables– Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. CON: intact controls; Veh: GC(glucocorticoid)+Vehicle; Ris: GC+Risedronate; Cal: GC+Calcitriol. a, significant vs CON; b, significant vs Veh; c, significant vs Ris. BV/TV: bone volume/total tissue volume; Tb N: trabecular number; Tb Th: trabecular thickness; Tb Sp: trabecular separation.

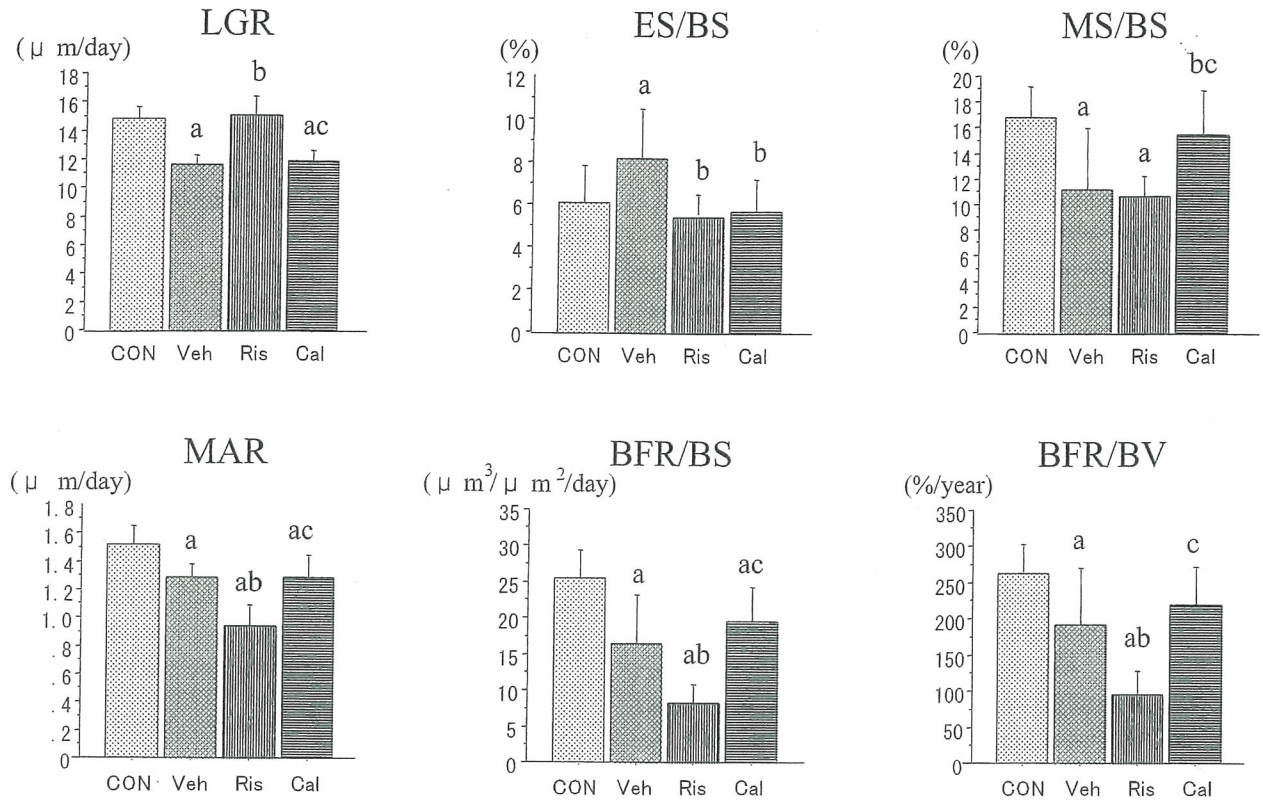

Fig. 2. Bone histomorphometric analysis of cancellous bone of the proximal tibial metaphysis –Formative and resorptive variables– Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. CON: intact controls; Veh: GC(glucocorticoid)+Vehicle; Ris: GC+Risedronate; Cal: GC+Calcitriol. a, significant vs CON; b, significant vs Veh; c, significant vs Ris. LGR: longitudinal growth rate; ES: eroded surface; BS: bone surface; MS: mineralizing surface; MAR: mineral apposition rate; BFR: bone formation rate; BV: bone volume.

bone growth and femoral bone length, and reduces the mechanical strength of the lumbar spine [5, 6, 15–17, 22]. GC-induced osteopenia has been associated with decreased bone formation, with the effects on bone resorption in terms of osteoclastic activity being inconsistent. Bone resorption has been reported to be affected more severely by higher doses of GCs [10], suggesting that higher daily or total doses of GC may decrease osteoclastic activity. Thus, the inconsistent effects on bone resorption observed may have been due to the inconsistent daily and total doses of GC. The key histological feature of corticosteroid-induced cancellous bone loss is reduction in Tb Th, reflecting suppressed bone formation [12].

In our study, the high-dose GC administration decreased cancellous BV/TV, Tb N, and Tb Th, primarily due to decreased bone formation and increased bone resorption. The GC administration also decreased LGR and tibial length. These results suggest that in the early (4 weeks) phase of the high-dose GC administration, bone resorption is increased and bone formation is decreased, resulting in cancellous osteopenia and a decrease in the longitudinal growth of long bones. Because 4-month-old rats were used, growth-related alterations of cancellous bone needed to be considered.

The efficacy of Ris or Cal against cancellous bone loss in ovariectomized rats has been well documented. Ris was shown to suppress bone resorption and prevent cancellous bone loss in ovariectomized rats [4, 18], and Cal with calcium supplementation was shown to decrease bone resorption and increase cancellous bone mass in ovariectomized osteopenic rats [12]. However, very few studies have reported the effect of Ris or Cal on cancellous bone mass in GC-treated rats.

In our study, Ris treatment markedly increased cancellous BV/TV and Tb N above the control level. These

effects were primarily due to suppressed bone turnover. On the other hand, Cal treatment attenuated the GC-induced decrease in cancellous BV/TV and Tb Th. These effects were primarily due to suppressed bone resorption and maintained or even increased bone formation. Thus, the differential effects of Ris and Cal on cancellous osteopenia were shown in GC-treated rats.

As discussed above, increased bone resorption and suppressed bone formation contributes to GC-induced bone loss. In addition, suppression of intestinal calcium absorption and decreased renal tubular calcium reabsorption with increased urinary calcium excretion have also been reported to contribute partly to GC-induced bone loss [7]. In our study, renal and intestinal calcium losses were not evaluated; however GC administration decreased serum calcium levels, suggesting that it might also suppress intestinal calcium absorption and renal tubular calcium reabsorption. Ris treatment accelerated the GC-induced decrease in serum calcium levels, probably because of accumulation in the bone of calcium from serum. However, Cal failed to increase serum calcium levels. Higher doses of Cal and/or much more calcium supplementation might be needed for serum calcium levels to increase.

Ris treatment prevented GC-induced deceases in longitudinal bone growth and tibial length. This result suggests that Ris does not adversely affect longitudinal bone growth in rats treated with GC. However, Cal treatment did not show any effect on tibial length, as previously reported [10].

Limitations should be noted. The duration of the study (4 weeks) seems to be too short. However, evidence suggests that the loss of BMD is more evident in cancellous bone than in cortical bone [23], and that high-dose GC therapy induces the rapid loss of BMD [24]. Actually, the present study did confirm that the high-dose GC administration induced cancellous osteopenia and deterioration of cancellous microarchitecture, as a result of increased bone resorption and decreased bone formation, and showed the differential effect of Ris and Cal on cancellous microarchitecture and bone formation and resorption in the early phase (4 weeks) of the high-dose GC administration. Further studies with longer duration of observation would be of interest to determine the effect of Ris and Cal on cortical bone as well as bone mechanical strength in GC-treated rats.

In conclusion, the present study showed the differential effects of Ris and Cal on cancellous osteopenia in rats treated with high-dose GC. The effects of Ris on cancellous osteopenia were associated with suppressed bone turnover, while those of Cal were associated with suppressed bone resorption and maintained or even increased bone formation.

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Comparative Effects of Alendronate and Alfacalcidol on Cancellous and Cortical Bone Mass and Bone Mechanical Properties in Ovariectomized Rats

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Abstract: *The purpose of the present study was to compare the effects of alendronate and alfacalcidol on cancellous and cortical bone mass and bone mechanical properties in ovariectomized rats. Twenty-six female Sprague-Dawley rats, 7 months of age, were randomized by the stratified weight method into four groups: the sham-operated control (Sham) group and the three ovariectomy (OVX) groups, namely, OVX + vehicle, OVX + alendronate (2.5 mg/kg, p.o., daily), and OVX + alfacalcidol (0.5 μg/kg, p.o., daily). At the end of the 8-week experimental period, bone histomorphometric analyses of cancellous bone at the proximal tibial metaphysis and cortical bone at the tibial diaphysis were performed, and the mechanical properties of the femoral distal metaphysis and femoral diaphysis were evaluated. OVX decreased cancellous bone volume per total tissue volume (BV/TV), and the maximum load of the femoral distal metaphysis, as a result of increases in serum osteocalcin (OC) levels, and also the number of osteoclasts (N.Oc), osteoclast surface (OcS) and bone formation rate (BFR) per bone surface (BS), and BFR/BV, without any effect on cortical area (Ct Ar), or maximum load of the femoral diaphysis. Alendronate prevented this decrease in cancellous BV/TV by suppressing increases in N.Oc/BS, OcS/BS, BFR/BS, and BFR/BV, without any apparent effect on Ct Ar, or maximum load of the femoral distal metaphysis and femoral diaphysis. On the other hand, alfacalcidol increased cancellous BV/TV, Ct Ar, and the maximum load of the femoral distal metaphysis and femoral diaphysis, by mildly decreasing trabecular BFR/BV, maintaining trabecular mineral apposition rate and osteoblast surface per BS, increasing periosteal and endocortical BFR/BS, and preventing an increase in endocortical eroded surface per BS. The present study clearly showed the differential skeletal effects of alendronate and alfacalcidol in ovariectomized rats. Alendronate prevented OVX-induced cancellous bone loss by*

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suppressing bone turnover, while alfacalcidol improved cancellous and cortical bone mass and bone strength by suppressing bone resorption and maintaining or even increasing bone formation.

Key words: *alendronate, alfacalcidol, osteopenia, ovariectomy, rat*

Introduction

Alendronate (a bisphosphonate, anti-resorptive agent) and alfacalcidol (active vitamin D_3) are widely used for the treatment of postmenopausal osteoporosis in Japan. The results of randomized controlled head-to-head trials suggest that alendronate (5 mg/day) is more effective than alfacalcidol (1 μg/day) in increasing lumbar bone mineral density (BMD) and reducing the incidence of vertebral fractures in Japanese postmenopausal women with osteoporosis [14, 23]. However, their effects on BMD and the incidence of fractures of skeletal sites rich in cortical bone remain uncertain.

Several preclinical studies have reported the efficacy of alendronate and alfacalcidol against osteopenia using a rat model of postmenopausal women. Alendronate suppresses bone turnover and prevents cancellous bone loss, or even increases cancellous bone mass, in ovariectomized rats [6, 9, 20]. On the other hand, alfacalcidol suppresses bone resorption, yet maintains or even stimulates bone formation, as reflected by increases in serum osteocalcin levels and bone formation rate at both cancellous and cortical bone sites, thereby increasing BMD and improving the mechanical properties of the bone [21]. However, very few studies have reported on the comparative effects of alendronate and alfacalcidol on both the bone mass and mechanical properties of skeletal sites rich in cancellous or cortical bone in ovariectomized rats. The purpose of the present study was to compare the effects of alendronate and alfacalcidol on cancellous and cortical bone mass and bone mechanical properties in ovariectomized rats.

Materials and Methods

Treatment of animals

Twenty-six female Sprague-Dawley rats, 7 months of age, were purchased from Charles River Japan (Kanagawa, Japan). They were fed a pelleted standard chow diet containing 1.25% calcium and 0.9% phosphorus (CRF-1: Oriental Kobo, Co., Ltd., Tokyo, Japan). The animals were housed under local vivarium conditions (temperature 23.3°C, humidity 55%, and a 12-h on/off light cycle), with free access to water. After allowing one week for adaptation to the new environment, the rats were randomized by the stratified weight method into the following four groups: the sham-operation + vehicle (Sham) group (n=5), and the three bilateral ovariectomy (OVX) groups, namely, OVX + vehicle (n=5), OVX + alendronate (2.5 mg/kg, n=8), and OVX + alfacalcidol (0.5 μg/kg, n=8). The treatment with vehicle, alendronate, or alfacalcidol was started one day after the surgery and continued for 8 weeks. Bilateral OVX was performed under general anesthesia induced by intraperitoneal injection of 25–30 mg/kg pentobarbital sodium. Tablet forms of alendronate (Bonalon, Teijin Pharma, Tokyo, Japan) or alfacalcidol (One-alfa, Teijin Pharma, Tokyo, Japan) were pulverized, dissolved in 0.1 ml of sterile saline, and administered orally to the animals (the OVX + alendronate and OVX + alfacalcidol groups, respectively) every day by gavage deep into the mouth. Vehicle (0.1 ml of sterile saline) was also administered orally to the animals (the Sham + vehicle and OVX + vehicle groups) every day by gavage deep into the mouth. The dose of alendronate was determined based on the results of a previous study [1]. In OVX rats, 1.0 mg/kg of oral alendronate prevented OVX-induced cancellous bone loss in the proximal metaphysis, while 5.0 mg/kg of oral alendronate prevented OVX-induced BMD loss of the proximal femur. Thus, in the present study, 2.5 mg/kg of oral alendronate was adopted. The dose of alfacalcidol was determined so that the dose ratio of alfacalcidol to alendronate was 1 μg/5 mg based on the clinically available dose. This dose of alfacalcidol was considered to be effective, but was high according to the results of previous studies [10, 21, 22]. The body weight of the rats was monitored weekly. The present study was carried out at the laboratory of Hamri Co., Ltd. (Ibaraki, Japan). The animals

were maintained according to the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals, and the animal experimental protocols were approved by the Laboratory Animal Care Committee of Hamri Co., Ltd. (Ibaraki, Japan).

Preparation of specimens

All the rats were labeled with 25 mg/kg tetracycline (Sigma Chemical, St. Louis, MO, USA) injected intramuscularly and 8 mg/kg calcein (Sigma Chemical, St. Louis, MO, USA) injected subcutaneously at 9 days and 3 days, respectively, before sacrifice. The rats were sacrificed at 8 weeks after the start of the experiment. Before the animals were sacrificed, urine samples were collected over a 24-h period using metabolic cages, and the specimens were stored at –20°C. The animals were sacrificed by exsanguination after being anesthetized by intraperitoneal injection of 25–30 mg/kg pentobarbital sodium. Serum specimens were collected from all the rats, and the right femur and right tibia were isolated.

The serum samples were stored at –20°C. The urine and serum samples were used for the measurements of the biochemical markers as described below. The femurs were stored at –20°C and then used for biomechanical testing as described below. The tibiae were processed for bone histomorphometric analyses. The bones were fixed in cold 40% ethanol overnight, and then cut into three parts using an Isomet saw (Buehler, Lake Bluff, IL, USA). The proximal tibial metaphysis and tibial diaphysis with the fibular junction were stained with Villanueva Osteochrome Bone Stain (Polyscience, Warrington, PA, USA) for 5 days. The specimens were dehydrated sequentially in ascending concentrations of ethanol (70%, 95%, and 100%) and xylene, and then embedded in methyl-methacrylate (EM Science, Gibbstown, NJ, USA) at 4°C according to the method of Erben [7]. Cross-sections of the tibial diaphysis just proximal to the tibio-fibular junction were cut at 40 μm thickness using a diamond wire Histo-Saw machine (Delaware Diamond Knives, Wilmington, DE, USA), and the thickness of each cross-sectional specimen was determined with an Inspectors' Dial Bench Gauge (L.S. Starrett, Athol, MA, USA). Frontal sections of the proximal tibial metaphysis were cut at 8 μm or 4 μm thickness using a microtome (Leica RM2155; Leica Inc., Nussloch, Germany). The 8-μm sections were then transferred onto chromalum-gelatin-coated slides and dried overnight under a press at 42°C. All the sections were coverslipped with Eukitt (Calibrated Instruments, Hawthorne, NY, USA) for the static and dynamic histomorphometric analyses. For tartrate-resistant acid phosphatase (TRAP) histochemistry, 8-μm sections of the proximal tibial metaphysis were deplasticized with three changes of 2-methoxyethylacetate for 30 min each, two changes of acetone for 5 min each, and sequential changes of ethanol (95%, 70%, and 40%), and finally, two changes of deionized water for 5 min each for rehydration. The deplasticized and rehydrated sections (8 μm thickness) were placed in 0.1 M acetate buffer at pH 5.0 for 5 min, and the TRAP reaction was subsequently performed using a leukocyte acid phosphatase kit (Sigma Chemical, St. Louis, MO, USA). The sections stained for TRAP were counterstained using Mayer's hematoxylin (1 min) and the sections were air-dried and mounted with a plastic UV mounting medium (Polysciences Inc., Warrington, PA, USA). For Goldner Trichrom staining to count the osteoblast surface, adjacent 4-μm sections of the proximal tibia metaphysis were deplasticized and rehydrated, followed by the procedure of Goldner Trichrom stain, then mounted with Eukitt (Calibrated Instruments, Hawthorne, NY, USA).

Urine and serum biochemical analyses

The levels of urinary deoxypyridinoline (DPD) as a bone resorption marker were measured by enzyme-immunoassay (EIA) using a Pyrilinks-D kit (Metra Biosystems Inc., CA, USA). The serum calcium and phosphorus levels were measured by the o-CPC and ammonium molybdate colorimetric methods, respectively, using an autoanalyzer (Dada Behring Model RXL, Bakersfield, CA, USA). The levels of serum osteocalcin (OC) as a bone formation marker were measured by immunoradiometric assay (IRMA) using a Rat Osteocalcin IRMA kit (Immutopics, Inc., CA, USA).

Biomechanical testing

The mechanical properties of the femoral diaphysis were evaluated by the three-point bending test. Load was applied midway between two supports placed 15 mm apart on the bone. The femur was positioned so that the loading point was at the center of the femoral diaphysis and bending occurred about the medial-lat-

eral axis. The specimens were tested in a saline bath at 37°C. Each specimen was submerged in the saline bath for about 3 min before the testing, to allow temperature equilibration. Load-displacement curves were recorded at a crosshead speed of 20 mm/min using a materials-testing machine (MZ500D; Maruto, Co., Ltd., Tokyo, Japan). The parameters analyzed were the maximum load, stiffness, and breaking energy.

Just after the three-point bending test of the femoral diaphysis, the distal metaphysis of the femur was isolated over a length of 10 mm from the joint surface of the femoral condyle. The mechanical properties of this segment were then measured by the compression test. Compressive load was applied by the rectangular parallelepiped crosshead (length 2 cm, width 2 cm, and height 1 cm) on the femoral distal metaphysis from the lateral aspect to the medial aspect. The specimens were positioned so that the loading point was at the center of the femoral lateral condyle. The specimens were tested in a saline bath at 37°C. Each specimen was submerged in the saline bath for about 3 min before the testing, to allow temperature equilibration. Load-displacement curves were recorded at a crosshead speed of 10 mm/minute and compression depth of 2.5 mm, using a materials-testing machine (MZ500D; Maruto, Co., Ltd., Tokyo, Japan). The parameters analyzed were maximum load, stiffness, and breaking energy.

Bone histomorphometry of the tibia

A digitizing morphometry system was used to measure bone histomorphometric parameters. The system consisted of an epifluorescence microscope (Nikon E-400, OsteoMetrics, Atlanta, GA, USA), an Osteomeasure High Resolution Color Subsystem (OsteoMetrics, Atlanta, GA, USA), and a digitizing pad (Numonics 2206; Numonics Corp., Montomerville, PA, USA) coupled to an IBM computer, and a morphometry program (OsteoMetrics, Atlanta, GA, USA). The measured parameters for cancellous bone included total tissue volume (TV), bone volume (BV), bone surface (BS), eroded surface (ES), single- and double-labeled surfaces (sLS and dLS, respectively), and osteoblast surface (ObS). These data were used to calculate percent cancellous bone volume (BV/TV), trabecular number (Tb N), trabecular thickness (Tb Th), trabecular separation (Tb Sp), ES/BS, MS/BS [(sLS/2+dLS)/BS], mineral apposition rate (MAR), bone formation rate (BFR)/BS, BFR/BV, and ObS/BS, in accordance with the standard nomenclature described by Parfitt *et al.* [17]. In the present study, the region of cancellous bone measured was 1–4 mm distal to the lower margin of the growth plate in the proximal tibia, which consists of secondary spongiosa. Cells showing positive staining for TRAP were counted in the region from the distal end of the growth plate to 0.2 mm from the growth plate, and the number of osteoclasts (N.Oc) and osteoclast surface (OcS) per BS were calculated. The measured parameters for cortical bone were total tissue area (Tt Ar), marrow area (Ma Ar), endocortical ES, periosteal and endocortical BS, sLS, dLS, and interlabel width. These data were used to calculate cortical bone area (Ct Ar), endocortical ES/BS, and periosteal and endocortical MS/BS [(sLS/2+dLS)/BS], MAR, and BFR/BS.

Statistical analysis

All the data was expressed as means and standard deviation (SD). Multiple comparisons of data among the groups were performed by analysis of variance (ANOVA) with Fisher's protected least significant difference (PLSD) test. All statistical analyses were performed using the Stat View J-5.0 program on a Macintosh computer. A significance level of $P<0.05$ was used for all the comparisons.

Results

Body weight and biochemical markers (Table 1 and Fig. 1)

The initial body weight did not differ significantly among the four groups. OVX was associated with an increase in the body weight of the animals. Neither alendronate nor alfacalcidol affected the body weight of the ovariectomized animals.

OVX increased the serum OC and urinary DPD levels, and decreased the serum calcium levels. Alendronate prevented the elevation of the urinary DPD level. On the other hand, alfacalcidol enhanced the elevation of both the markers and also increased the serum phosphorus level.

Bone histomorphometic analysis of the cancellous bone of the proximal tibial metaphysis (Fig. 2 and Table 2)

OVX decreased cancellous BV/TV and Tb N and

Table 1. Body weight and serum calcium and phosphorus

	Initial body weight (g)	Final body weight (g)	Calcium (mg/dl)	Phosphorus (mg/dl)
Sham	364 ± 33	341 ± 22	10.5 ± 0.7	5.5 ± 0.9
OVX				
Vehicle	364 ± 29	404 ± 26[a]	9.6 ± 0.5[a]	5.7 ± 0.5
Alendronate	363 ± 35	380 ± 26[a]	9.4 ± 0.3[a]	5.2 ± 0.3
Alfacalcidol	369 ± 29	378 ± 23[a]	10.0 ± 0.2[ac]	7.0 ± 0.5[abc]

Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. [a]: significant vs Sham, [b]: significant vs Vehicle, [c]: significant vs Alendronate.

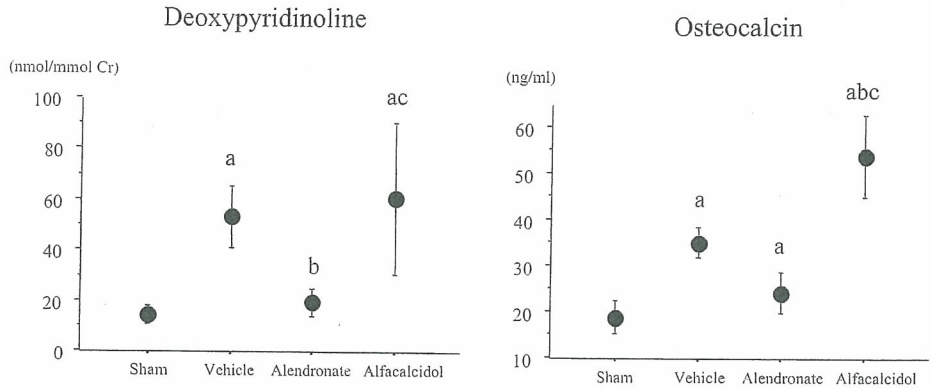

Fig 1. Bone markers Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. a: significant vs Sham, b: significant vs Vehicle, c: significant vs Alendronate.

increased Tb Sp, as a result of increased bone resorption (N.Oc/BS, OcS/BS) and bone formation (ObS/BS, MS/BS, MAR, BFR/BS, BFR/BV). Alendronate prevented these changes of the structural parameters, primarily by suppressing bone resorption (ES/BS, N.Oc/BS, OcS/BS) and bone formation (ObS/BS, MS/BS, MAR, BFR/BS, BFR/BV). However, suppression of bone formation (MS/BS, BFR/BS, BFR/BV) was marked. Alfacalcidol increased cancellous BV/TV and Tb Th to beyond the values obtained in the sham-operated controls, and prevented the alterations of Tb N and Tb Sp, primarily by mildly suppressing bone resorption (ES/BS, N.Oc/BS, OcS/BS) and bone formation (MS/BS, BFR/BS, BFR/BV). The effect of alfacalcidol on cancellous BV/TV was greater than that of alendronate, because the decreases of MS/BS, BFR/BS, and BFR/BV induced by alfacalcidol were comparatively mild, and ObS/BS and MAR were maintained by alfacalcidol.

Bone histomorphometric analysis of the cortical bone of the tibial diaphysis (Fig. 3 and Table 3)

OVX did not affect Tt At, Ct Ar, or Ma Ar, despite increased periosteal bone formation (MS/BS, MAR, BFR/BS) and endocortical bone resorption (ES/BS). Alendronate did not affect Tt At, Ct Ar or Ma Ar, despite suppressed endocortical bone resorption (ES/BS). Alfacalcidol increased Tt At and Ct Ar as compared with the values in the OVX controls, as a result of increased periosteal and endocortical bone formation (MS/BS, MAR, BFR/BS).

Biomechanical test of the femur (Fig. 4)

OVX decreased the maximum load and stiffness of the femoral distal metaphysis, without any effect on the mechanical properties of the femoral diaphysis. Alendronate did not affect any of the mechanical properties of the femoral distal metaphysis or diaphysis,

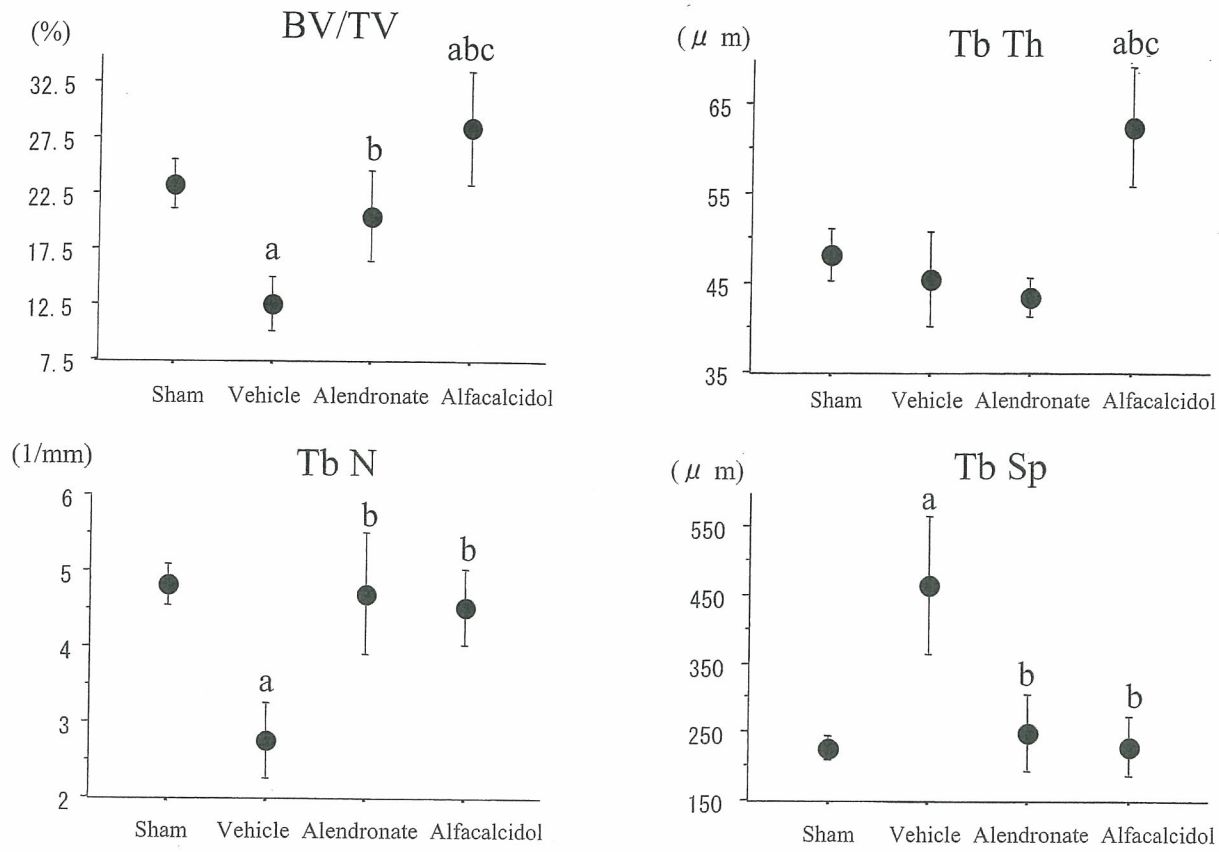

Fig 2. Bone histomorphometric analysis of the cancellous bone of the proximal tibial metaphysis. –Structural parameters– Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. a: significant vs Sham, b: significant vs Vehicle, c: significant vs Alendronate. BV/TV: bone volume/total tissue volume, Tb N: trabecular number, Tb Th: trabecular thickness, Tb Sp: trabecular separation.

Table 2. Histomorphometric analysis of the cancellous bone of the proximal tibial metaphysis –Formative and resorptive variables–

	ES/BS (%)	N.Oc/BS (#/mm)	OcS/BS (%)	ObS/BS (%)	MS/BS (%)	MAR (μm/day)	BFR/BS (μm³/μm²/day)	BFR/BV (%/year)
Sham	11.6 ± 3.7	1.75 ± 0.10	5.4 ± 0.7	10.4 ± 2.4	13.3 ± 2.0	0.44 ± 0.05	5.8 ± 0.8	74 ± 12
OVX								
Vehicle	11.5 ± 2.8	4.12 ± 1.05[a]	14.1 ± 3.0[a]	17.7 ± 3.7[a]	29.5 ± 4.0[a]	0.75 ± 0.14[a]	22.3 ± 6.3[a]	297 ± 68[a]
Alendronate	5.8 ± 1.6[ab]	1.66 ± 0.29[b]	5.3 ± 1.0[b]	14.1 ± 2.1[ab]	8.3 ± 2.3[ab]	0.44 ± 0.10[b]	3.7 ± 1.4[b]	52 ± 22[b]
Alfacalcidol	8.4 ± 1.6[abc]	1.99 ± 0.35[b]	7.1 ± 1.3[bc]	18.8 ± 2.8[ac]	22.0 ± 4.2[abc]	0.70 ± 0.06[ac]	15.5 ± 3.4[abc]	152 ± 35[abc]

Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. [a]: significant vs Sham, [b]: significant vs Vehicle, [c]: significant vs Alendronate. ES: eroded surface, BS: bone surface, N.O c: number of osteoclast, ObS: osteoblast surface, MS: mineralizing surface, MAR: mineral apposition rate, BFR: bone formation rate, BV: bone volume.

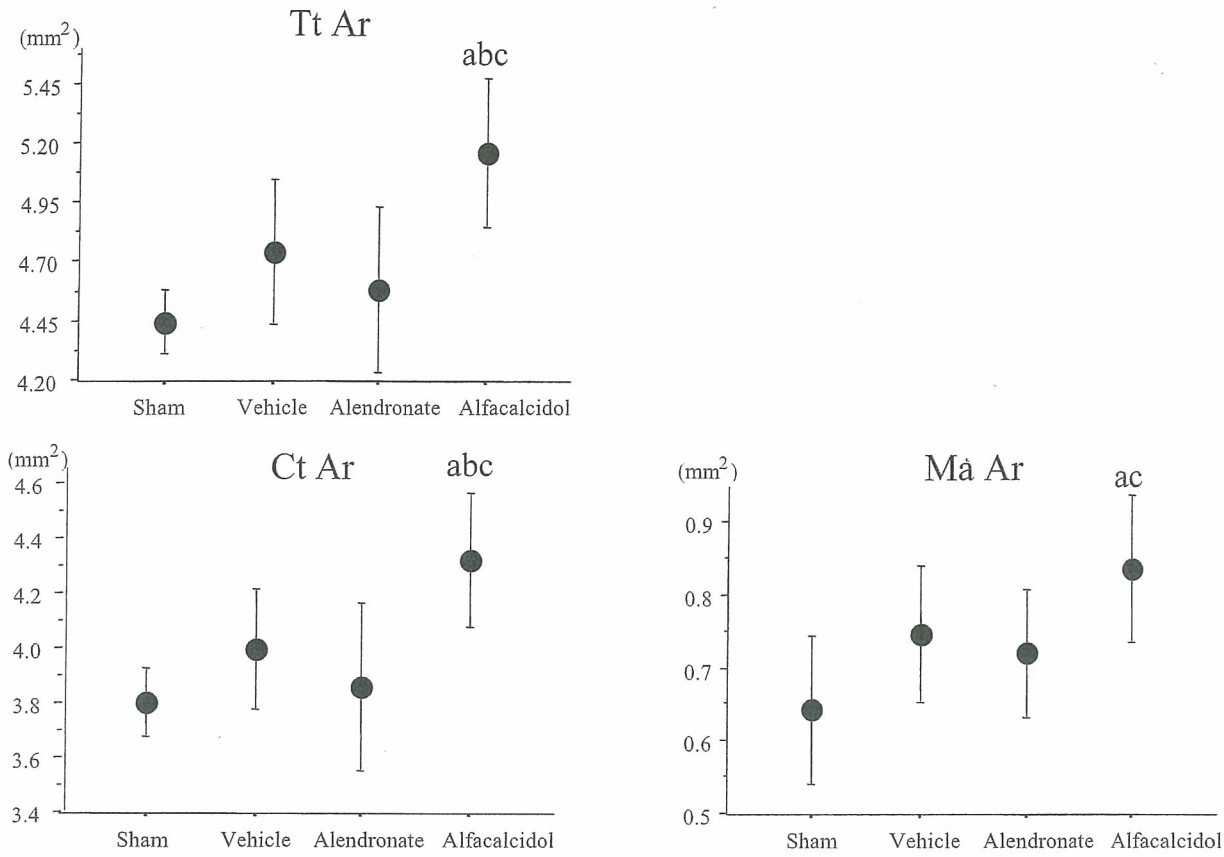

Fig 3. Bone histomorphometric analysis of the cortical bone of the tibial diaphysis. –Structural parameters– Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. a: significant vs Sham, b: significant vs Vehicle, c: significant vs Alendronate. Tt Ar: Total tissue area, Ct Ar: cortical area, Ma Ar: marrow area.

Table 3. Histomorphometric analysis of the cortical bone of the tibial diaphysis –Formative and resorptive variables–

	Periosteal			Endocortical			
	MS/BS (%)	MAR (μm/day)	BFR/BS (μm³/μm²/day)	ES/BS (%)	MS/BS (%)	MAR (μm/day)	BFR/BS (μm³/μm²/day)
Sham	4.9 ± 4.5	0.86 ± 0.11	4.5 ± 5.0	41.0 ± 9.2	4.9 ± 0.8	0.16 ± 0.22	0.82 ± 1.2
OVX							
Vehicle	61.6 ± 19.0[a]	1.18 ± 0.20[a]	73.0 ± 24.0[a]	62.3 ± 9.4[a]	11.4 ± 5.2[a]	0.53 ± 0.17	5.65 ± 2.00
Alendronate	59.9 ± 15.5[a]	1.11 ± 0.25[a]	67.7 ± 23.9[a]	36.7 ± 11.3[b]	10.3 ± 0.5	0.28 ± 0.36	2.72 ± 3.81
Alfacalcidol	80.2 ± 11.5[abc]	1.58 ± 0.19[abc]	127.5 ± 25.9[abc]	38.4 ± 11.3[b]	26.1 ± 12.6[abc]	1.34 ± 0.29[abc]	35.80 ± 19.18[abc]

Data are expressed as mean ± SD. ANOVA with Fisher's PLSD test was used to compare the data among the groups. [a]: significant vs Sham, [b]: significant vs Vehicle, [c]: significant vs Alendronate. MS: mineralizing surface, BS: bone surface, MAR: mineral apposition rate, BFR: bone formation rate, ES: eroded surface.

whereas alfacalcidol increased the maximum load and breaking energy of the femoral distal metaphysis and the maximum load and stiffness of the femoral diaphysis beyond the values obtained in the sham-operated controls.

Discussion

The present study demonstrated that alendronate prevented the decrease in cancellous BV/TV induced by OVX by suppressing bone turnover without any effect