

# Alfacalcidol prevents age-related bone loss and causes an atypical pattern of bone formation in aged male rats

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## Abstract

The current study was designed to investigate the skeletal effects of alfacalcidol in aged rats. Eighteen-month-old male rats were treated with 0, 0.1, or 0.2 µg/kg/d of alfacalcidol by daily oral gavage, 5 days/week for 12 weeks. At the beginning of the treatments, one group of rats was euthanized to serve as a baseline control. At the end of the study, the second lumbar vertebrae and the right tibial diaphyses were processed for bone histomorphometric analysis. The fourth lumbar vertebrae were subjected to strength testing. The control group of rats at 21 months of age had decreased serum testosterone levels and decreased cancellous bone mass associated with increased bone turnover on the trabecular surface. The older rats had increased bone turnover on the endocortical surface and decreased bone formation on the periosteal surface compared with the 18-month group. In contrast, alfacalcidol treatment increased cancellous and cortical bone mass in aged male rats. Trabecular bone resorption was decreased whereas bone formation was maintained or increased in the rats treated with alfacalcidol. In addition, endocortical bone formation was decreased whereas periosteal bone formation was increased in the rats treated with alfacalcidol compared with vehicle-treated rats. Marrow trabecular bone area was increased by alfacalcidol treatment in tibial diaphyses. Furthermore, bone strength of the lumbar vertebral body was increased after alfacalcidol treatment. An atypical pattern of bone formation on endosteal bone surfaces was seen in the rats treated with alfacalcidol. The atypical bone formation is characterized by small, focal packets of newly formed bone on trabecular and endocortical bone surfaces. This gave the appearance of the formation of "bone buds" emanating from trabecular surfaces. These bony outgrowths were mineralized and demonstrated significant fluorochrome label indicating recent mineralization. Also, lamellae of the bone buds did not run parallel to those of the trabecular plate to which they are attached. Arrest lines presented in most of the "bone buds". In summary, alfacalcidol treatment increased cancellous and cortical bone mass and improved bone strength, resulting in the prevention of age-related bone loss in aged male rats. An atypical pattern of bone formation observed in this study may be a result of minimodeling based bone formation stimulated by alfacalcidol treatment.

**Keywords:** Alfacalcidol, Bone Formation, Bone Resorption, Bone Mass, Bone Strength

## Introduction

Vitamin D is an essential factor for the development and maintenance of skeleton. In humans, calcitriol (1 $\alpha$ ,25-dihy-

droxyvitamin D<sub>3</sub> or 1,25(OH)<sub>2</sub>D<sub>3</sub>) and its prodrug, alfacalcidol (1 $\alpha$ -hydroxyvitamin D<sub>3</sub> or 1 $\alpha$ (OH)D<sub>3</sub>) reduce vertebral and hip fractures in postmenopausal and senile osteoporosis, as well as in glucocorticoid-induced osteoporosis<sup>1-9</sup>. However, the mechanism of action of the active vitamin D metabolites on bone as it contributes to the fracture reduction remains unclear. *In vitro* and *in vivo* studies have shown that calcitriol has stimulatory effects on osteoclastogenesis and osteoclast activity<sup>10-14</sup>. This effect on osteoclasts is believed to be mediated through osteoblastic cells<sup>15,16</sup>. However, the effects of calcitriol on bone resorption are counteracted *in vivo* by suppression of parathyroid hormone (PTH) secretion through both a direct inhibitory effect on the parathyroid glands and an indirect effect via stimulation of intestinal calcium absorption and a subsequent rise in serum calcium<sup>17</sup>.

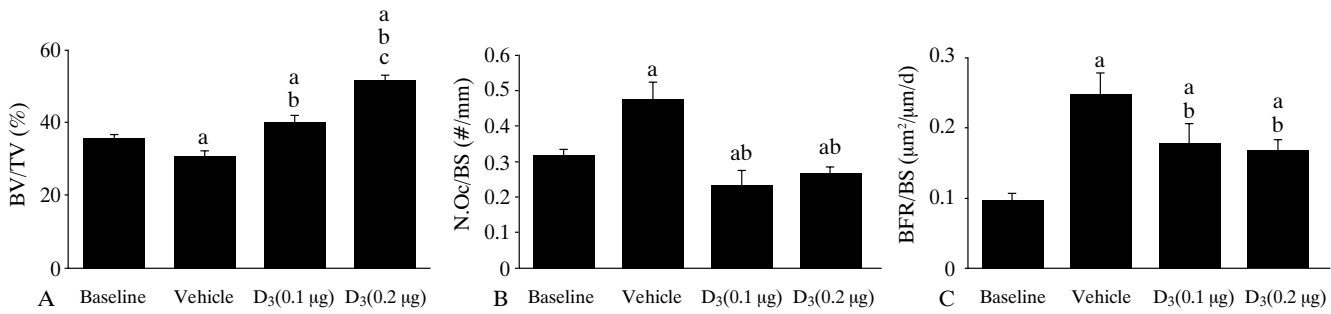
In recent years, there is increasing evidence to support the

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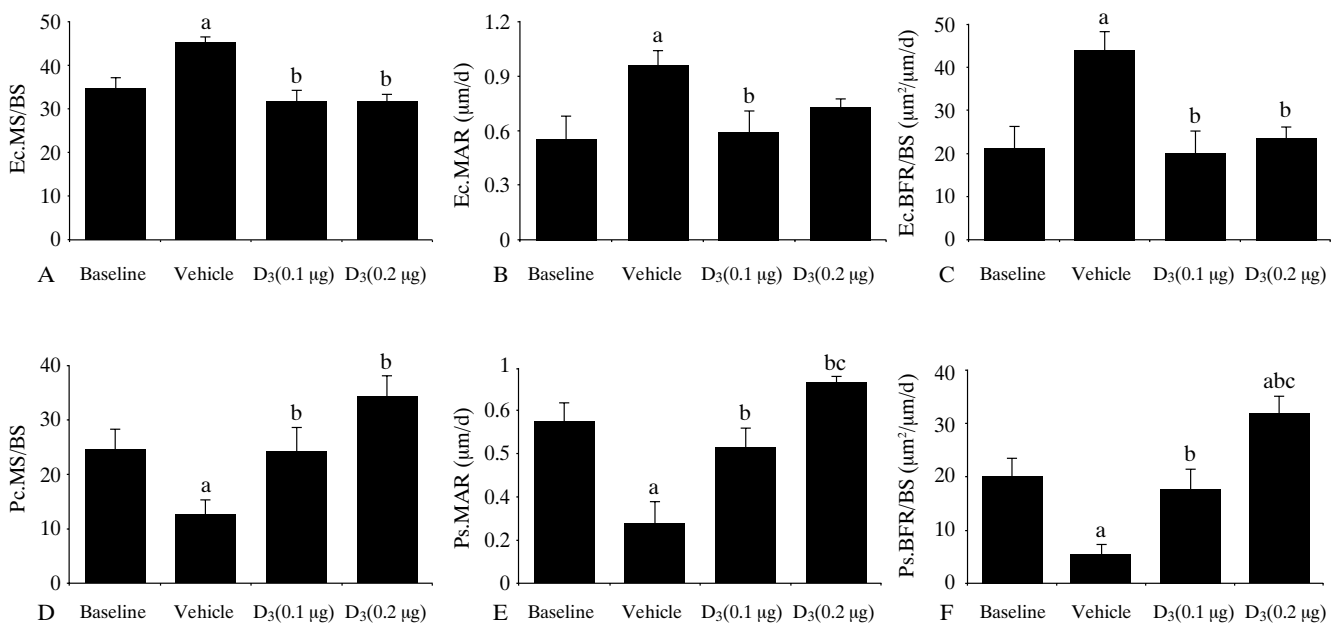
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**Figure 1.** Cancellous bone volume (BV/TV), osteoclast number (N.OC/BS), and surface-based bone formation rate (BFR/BS) of 2<sup>nd</sup> lumbar vertebrae of baseline rats and the rats treated with either vehicle or alfacalcidol at 0.1 or 0.2 µg/kg/d, 5 days/week, for 12 weeks. Data are expressed as mean ± SEM. <sup>a</sup>*p* < 0.05 vs. baseline; <sup>b</sup>*p* < 0.05 vs. vehicle; <sup>c</sup>*p* < 0.05 vs. low dose alfacalcidol.



**Figure 2.** Dynamic cortical bone histomorphometric variables of the tibial diaphyses of baseline rats and the rats treated with either vehicle or alfacalcidol at 0.1 or 0.2 µg/kg/d, 5 days/week, for 12 weeks. Ec.MS/BS: endocortical mineralizing surface; Ec.MAR: endocortical mineral apposition rate; Ec.BFR/BS: endocortical bone formation rate; Ps.MS/BS: periosteal mineralizing surface; Ps.MAR: periosteal mineral apposition rate; Ps.BFR/BS: periosteal bone formation rate. Data are expressed as mean ± SEM. <sup>a</sup>*p* < 0.05 vs. baseline; <sup>b</sup>*p* < 0.05 vs. vehicle; <sup>c</sup>*p* < 0.05 vs. low dose alfacalcidol.

notion that vitamin D may effectively stimulate osteoblastic activity and exert an anabolic effect on bone. Osteoblasts, the cells that are responsible for bone formation, possess abundant vitamin D receptors<sup>18,19</sup>. Studies in vitamin D receptor (VDR) knockout mice revealed that the 1,25(OH)<sub>2</sub>D<sub>3</sub>-VDR complex was essential for bone growth and bone formation through its physiological effect on intestinal calcium absorption<sup>20,21</sup>. In addition, *in vitro* studies showed that calcitriol stimulated the synthesis of a variety of noncollagenous proteins such as osteocalcin, matrix Gla protein, osteopontin, fibronectin, as well as alkaline phosphatase activity in osteoblast model systems<sup>22-30</sup>. In intact rats, high doses of calcitriol up-regulated tibial osteocalcin messenger RNA levels<sup>31</sup>

and increased the number of osteoblast precursor cells in bone marrow of tibia as well as stimulated cancellous bone formation in lumbar vertebra<sup>32</sup>. Furthermore, calcitriol and alfacalcidol have been reported to stimulate bone formation and increase bone mass and strength in ovariectomized (OVX) rats<sup>33-40</sup>. These results suggest that active vitamin D stimulates osteoblastic activity and bone formation. However, the skeletal effects of alfacalcidol have not been assessed in aged animal models, which may be more relevant to elderly population. Therefore, the aim of this study was to investigate the effects of alfacalcidol on bone in aged male rats to further understand the relationship between changes in bone formation and bone mass as well as bone strength.

## Materials and methods

### *Animals and materials*

Eighteen-month-old male Sprague-Dawley rats (Harlan, Indianapolis, Indiana, USA) weighing an average of 540g were used in this study. The animals were housed at 24°C with a 12h light/12h dark cycle. They were allowed free access to water and were restricted to 28 grams/day of a commercial diet (Purina Laboratory Rodent Chow 5001, Purina-Mills, St. Louis, MO) containing 0.95% calcium, 0.67% phosphorus, and 4.5 IU/g vitamin D<sub>3</sub>. The experiments were conducted according to Pfizer animal care-approved protocols, and animals were maintained in accordance with the ILAR (Institute of Laboratory Animal Research) Guide for the Care and Use of Laboratory Animals.

Forty rats were randomly divided into 4 groups with 10 per group. One group of rats was euthanized at the beginning of the experiment to serve as baseline. The remaining rats were treated with vehicle alone (cottonseed oil) or alfacalcidol at 0.1 or 0.2 µg/kg body weight/d by daily oral gavage, 5 days/week for 12 weeks. The doses and dosing regimen used in this study were based on their efficacy shown in rats previously<sup>37-40</sup>. Alfacalcidol was purchased from Calbiochem, La Jolla, CA. Cottonseed oil, and calcein were purchased from Sigma Chemical Co., St. Louis, MO. The stock solution of alfacalcidol was made by dissolving alfacalcidol in 100% ethanol at a concentration of 0.1 mg/ml, protected from light, and stored at 4°C. The dosing solutions were prepared weekly by diluting the stock solution with a cottonseed oil vehicle to the concentration of 0.1 µg/ml and 0.2 µg/ml for the 0.1 µg/kg/d and 0.2 µg/kg/d group, respectively. All rats were injected subcutaneously with calcein on -12 and -2 days prior to necropsy at a dose of 10 mg/kg. This regimen resulted in deposition of a single or double fluorochrome label at bone surfaces that were actively mineralizing at the time of the injections. At the conclusion of 12 weeks of treatment with alfacalcidol, the animals were fasted 16 hours prior to necropsy. At the day of necropsy, whole blood samples were collected by cardiac puncture for biochemical assessments immediately after euthanasia by CO<sub>2</sub> asphyxiation. Blood samples were centrifuged to obtain the sera, which were stored at -20°C until assay. The 2<sup>nd</sup> lumbar vertebrae (LV<sub>2</sub>) and the right tibial diaphyses were harvested and prepared for bone histomorphometric analysis. The 4<sup>th</sup> lumbar vertebrae (LV<sub>4</sub>) were harvested and prepared for biomechanical test.

### *Serum biochemistry*

Serum calcium (Ca) and phosphate (P<sub>i</sub>) concentrations were measured with the Cobas Fara 2 analyzer (Roche Diagnostic System, Hoffman-La Roche Inc. Indianapolis, IN). Total serum testosterone levels were measured with a Coat-a-Count kit-TKTT1 (Diagnostic Products Corp., Los Angeles, CA). The limit of detection is 0.04 ng/ml. The cross reactivity is less than 5% for dihydrotestosterone and less than 1% for other steroids, including all major androgens and estrogens.

### *Cancellous bone histomorphometry of lumbar vertebrae*

The LV<sub>2</sub> were fixed in 10% formaldehyde for 24 hours and then dehydrated in graded concentration of ethanol and embedded undecalcified in methyl methacrylate<sup>41</sup>. Longitudinal frontal sections of lumbar vertebra were cut at 4- and 10-µm thickness using a Reichert-Jung Polycut S microtome (Leica Corp., Deerfield, IL, Heidelberg, Germany). One set of the 4-µm sections was stained with modified Masson's Trichrome stain for static histomorphometric measurements. Another set of the 4-µm sections was stained for cement lines by using surface-staining technique described by Erben<sup>42</sup>. The 10-µm sections remained unstained for the measurements of fluorochrome-based indices of bone formation.

Histomorphometric measurements were performed in cancellous bone tissue between 0.5 mm from the cranial and caudal growth plates of the lumbar vertebral body using an Image Analysis System (Osteomeasure, Inc., Atlanta, GA). Cancellous bone volume as a percentage of bone tissue area (BV/TV), osteoclast surfaces as percentages of total cancellous perimeter (Oc.S/BS), and osteoclast number per bone surface (N.Oc/BS) were measured in 4-µm thick, stained sections. Trabecular number (Tb.N), thickness (Tb.Th) and separation (Tb.Sp) were calculated as described by Parfitt et al.<sup>43</sup>. Fluorochrome-based indices of bone formation including the percentage of cancellous bone surface with a double fluorochrome label (mineralizing surface, MS/BS) and mineral apposition rate (MAR) were measured in 10-µm thick, unstained sections. In addition, bone formation rate (bone surface referent) was calculated by multiplying mineralizing surface by mineral apposition rate (BFR/BS). Values for mineral apposition rate were not corrected for obliquity of the plane of section in cancellous bone<sup>44</sup>.

### *Cortical bone histomorphometry of tibial diaphyses*

The tibial diaphyses were fixed in 70% ethanol, stained with Villanueva bone stain (Polysciences, Inc., Warrington, PA)<sup>45</sup>, dehydrated in ascending concentrations of ethanol, acetone, and embedded undecalcified in methyl methacrylate<sup>46</sup>. Cross-sections of tibial diaphyses (just proximal to the tibiofibular junction) were sawed to a 230-µm thickness using an isomet low speed saw (Buechler, Lake Bluff, IL) and then further ground by hand to about 30 µm for cortical histomorphometric measurements<sup>46</sup>.

Static histomorphometric variables measured in tibial diaphyses included total tissue area (T.Ar), percent cortical area (% Ct.Ar), percent marrow area (% Ma.Ar), cortical width (Ct.Wi), marrow trabecular bone area (Ma.Tb.Ar), and endocortical percent eroded surface (ES/BS). Dynamic histomorphometric variables included the percent mineralizing surface (Ec.MS/BS), mineral apposition rate (Ec.MAR), and surfaced-based bone formation rate (Ec.BFR/BS) on the endocortical surface and the percent mineralizing surface (Ps.MS/BS), mineral apposition rate (Ps.MAR), and surfaced-based bone formation rate (Ps.BFR/BS) on the periosteal surface.

Variables	Group			
	Baseline	Vehicle	1 $\alpha$ -D <sub>3</sub> (0.1 $\mu$ g)	1 $\alpha$ -D <sub>3</sub> (0.2 $\mu$ g)
Body Weight (g)	539 $\pm$ 9	556 $\pm$ 11	556 $\pm$ 17	546 $\pm$ 15
Testosterone (pg/ml)	82.9 $\pm$ 11.8	44.9 $\pm$ 6.5 <sup>a</sup>	36.0 $\pm$ 11.2 <sup>a</sup>	37.6 $\pm$ 6.0 <sup>a</sup>
Calcium (mg/dl)	12.4 $\pm$ 0.1	12.1 $\pm$ 0.1	12.7 $\pm$ 0.2 <sup>b</sup>	13.2 $\pm$ 0.2 <sup>abc</sup>
Phosphorus (mg/dl)	7.4 $\pm$ 0.3	6.6 $\pm$ 0.2	7.9 $\pm$ 0.3 <sup>b</sup>	8.3 $\pm$ 0.3 <sup>ab</sup>

Data are expressed as mean  $\pm$  SEM. Baseline: rats were euthanized at the beginning of treatments; Vehicle: vehicle-treated controls; 1 $\alpha$ -D<sub>3</sub> (0.1  $\mu$ g): rats treated with 0.1  $\mu$ g/kg/d of alfacalcidol; 1 $\alpha$ -D<sub>3</sub> (0.2  $\mu$ g): rats treated with 0.2  $\mu$ g/kg/d of alfacalcidol. <sup>a</sup> $p$  < 0.05 vs. baseline group; <sup>b</sup> $p$  < 0.05 vs. vehicle-treated controls; <sup>c</sup> $p$  < 0.05 vs. rats treated with 0.1  $\mu$ g/kg/d of alfacalcidol.

**Table 1.** Body weights and biochemical variables.

Variables	Group			
	Baseline	Vehicle	1 $\alpha$ -D <sub>3</sub> (0.1 $\mu$ g)	1 $\alpha$ -D <sub>3</sub> (0.2 $\mu$ g)
Tb.N (#/mm)	2.9 $\pm$ 0.1	2.7 $\pm$ 0.1	3.1 $\pm$ 0.1 <sup>b</sup>	3.4 $\pm$ 0.1 <sup>ab</sup>
Tb.Th ( $\mu$ m)	123.1 $\pm$ 3.4	113.6 $\pm$ 5.0	130.5 $\pm$ 3.2 <sup>b</sup>	154.8 $\pm$ 5.6 <sup>abc</sup>
Tb.Sp ( $\mu$ m)	221.7 $\pm$ 10.9	254.6 $\pm$ 12.1 <sup>a</sup>	194.6 $\pm$ 12.9 <sup>b</sup>	141.5 $\pm$ 6.6 <sup>abc</sup>
Oc.S/BS (%)	1.2 $\pm$ 0.1	1.8 $\pm$ 0.2 <sup>a</sup>	1.0 $\pm$ 0.2 <sup>b</sup>	1.1 $\pm$ 0.1 <sup>b</sup>
MS/BS (%)	9.3 $\pm$ 0.6	19.0 $\pm$ 1.7 <sup>a</sup>	14.0 $\pm$ 1.7 <sup>ab</sup>	13.0 $\pm$ 1.1 <sup>ab</sup>
MAR ( $\mu$ m/d)	1.2 $\pm$ 0.0	1.3 $\pm$ 0.0 <sup>a</sup>	1.3 $\pm$ 0.1	1.3 $\pm$ 0.0 <sup>a</sup>

Data are expressed as mean  $\pm$  SEM. Baseline: rats euthanized at the beginning of treatments; Vehicle: vehicle-treated controls; 1 $\alpha$ -D<sub>3</sub> (0.1  $\mu$ g): rats treated with 0.1  $\mu$ g/kg/d of alfacalcidol; 1 $\alpha$ -D<sub>3</sub> (0.2  $\mu$ g): rats treated with 0.2  $\mu$ g/kg/d of alfacalcidol. Tb.N: trabecular number; Tb.Th: trabecular thickness; Tb.Sp: trabecular separation; Oc.S/BS: osteoclast surface; MS/BS: mineralizing surface; MAR: mineral apposition rate.

<sup>a</sup> $p$  < 0.05 vs. baseline group; <sup>b</sup> $p$  < 0.05 vs. vehicle-treated controls; <sup>c</sup> $p$  < 0.05 vs. rats treated with 0.1  $\mu$ g/kg/d of alfacalcidol.

**Table 2.** Cancellous bone histomorphometric variables in lumbar vertebrae (LV<sub>2</sub>).

### Biomechanical properties

Mechanical properties of LV<sub>4</sub> were determined by a compression test<sup>47,48</sup> using an Instron Mechanical Testing Machine (Instron 4465 retrofitted to 5500, Instron Corporation, Canton, MA). The spinous process and posterior pedicle arch were first removed from LV<sub>4</sub> using a low-speed saw (Buechler, Lake Bluff, IL). Both cranial and caudal ends of epiphyses were also removed with the same saw to obtain a vertebral body specimen with two parallel surfaces and a height approximately equal to 4 mm. Width in the medial-lateral and anterior-posterior directions at both the cranial and caudal ends was measured using digital calipers. Values obtained from the two ends were recorded and the averaged value was used in the calculation of cross-

sectional area. The height of the vertebral body specimen was measured with the calipers. The specimen was then compressed to failure at a displacement rate of 6 mm/min using a 5-kN load cell. The load and displacement curve was recorded by testing machine software (Merlin II, Instron) from each test. The locations for maximum load at failure, stiffness and energy absorbed were selected manually from the load and displacement curve and then calculated by the machine software. The intrinsic properties, stress, elastic modulus and toughness were calculated from maximum load, stiffness, energy absorbed, cross-sectional area, and height with the following equations.

Machine measurements

Maximum load (unit: N) ( $F_u$ )

Stiffness (unit: N/mm) (S)

Energy absorbed (unit: mJ) (W)

Variables	Group			
	Baseline	Vehicle	1 $\alpha$ -D <sub>3</sub> (0.1 $\mu$ g)	1 $\alpha$ -D <sub>3</sub> (0.2 $\mu$ g)
T.Ar (mm <sup>2</sup> )	6.42 $\pm$ 0.14	6.62 $\pm$ 0.14	6.88 $\pm$ 0.21 <sup>a</sup>	6.88 $\pm$ 0.10 <sup>a</sup>
%Ct.Ar (%)	81.2 $\pm$ 0.3	79.0 $\pm$ 0.5 <sup>a</sup>	81.8 $\pm$ 0.8 <sup>b</sup>	82.9 $\pm$ 0.8 <sup>b</sup>
%Ma.Ar (%)	18.8 $\pm$ 0.3	21.0 $\pm$ 0.5 <sup>a</sup>	18.2 $\pm$ 0.8 <sup>b</sup>	17.1 $\pm$ 0.8 <sup>b</sup>
Ct.Wi ( $\mu$ m)	890.1 $\pm$ 11.3	873.0 $\pm$ 9.8	904.8 $\pm$ 17.1	922.0 $\pm$ 21.2 <sup>b</sup>
Ma.Tb.Ar ( $\mu$ m <sup>2</sup> )	0.0 $\pm$ 0.00	1.5 $\pm$ 0.00	38.6 $\pm$ 0.02	57.9 $\pm$ 0.02 <sup>ab</sup>
ES/BS (%)	2.4 $\pm$ 0.3	3.5 $\pm$ 0.3 <sup>a</sup>	1.5 $\pm$ 0.2 <sup>ab</sup>	1.1 $\pm$ 0.1 <sup>ab</sup>

Data are expressed as mean  $\pm$  SEM. Baseline: rats were euthanized at the beginning of treatments; Vehicle: vehicle-treated controls; 1 $\alpha$ -D<sub>3</sub> (0.1  $\mu$ g): rats treated with 0.1  $\mu$ g/kg/d of alfacalcidol; 1 $\alpha$ -D<sub>3</sub> (0.2  $\mu$ g): rats treated with 0.2  $\mu$ g/kg/d of alfacalcidol. T.Ar: total tissue area; %Ct.Ar: percent cortical area; %Ma.Ar: percent marrow area; Ct.Wi: cortical width; Ma.Tb.Ar: marrow trabecular bone area; ES/BS: percent eroded surface. <sup>a</sup>  $p < 0.05$  vs. baseline group; <sup>b</sup>  $p < 0.05$  vs. vehicle-treated controls; <sup>c</sup>  $p < 0.05$  vs. rats treated with 0.1  $\mu$ g/kg/d of alfacalcidol.

**Table 3.** Static histomorphometric variables in the tibial diaphyses.

#### Caliper measurements

- Width in the anterior-posterior direction (unit: mm) (a)
- Width in the medial-lateral direction (unit: mm) (b)
- Height of the vertebral body in the cranio-caudal direction (unit: mm) (h)

#### Derived parameters

- Cross-sectional area (unit: mm<sup>2</sup>) (CSA)
- Formula  $CSA = p * a * b/4$
- Stress (unit: N/mm) ( $\sigma$ )
- Formula  $\sigma = F_v/CSA$
- Elastic modulus (unit: MPa) (E)
- Formula  $E = S/(CSA/h)$
- Toughness (unit: MJ/m<sup>3</sup>) (T)
- Formula  $T = W/(CSA * h)$

#### Statistical analysis

Data are expressed as the mean  $\pm$  SEM for each group. Statistics were calculated using StatView 4.0 packages (Abacus Concepts., Inc., Berkeley, CA). Statistical differences between groups were evaluated with ANOVA followed by the Fisher PLSD test for multiple comparisons. Probabilities ( $p$ ) less than 0.05 were considered significant.

## Results

**Body weights and serum biochemistry.** The mean body weights of each group remained constant throughout the experimental period and were not significantly different from each other (Table 1). Serum testosterone was significantly lower in the rats at 21 months of age than at 18 months of age (Table 1). This parameter was not altered by alfacalcidol treatments. The rats treated with alfacalcidol demonstrated significantly increased serum calcium in a dose responsive manner when compared to those treated with vehicle (Table 1). Serum phosphorous levels were also significantly increased

in a dose responsive manner in rats treated with alfacalcidol as compared to those treated with vehicle (Table 1).

**Lumbar vertebrae (LV<sub>2</sub>).** Bone histomorphometric data from LV<sub>2</sub> are shown in Figure 1 and Table 2. Bone volume in the 21-month control rats was significantly decreased whereas trabecular separation, osteoclast surface, osteoclast number, mineralizing surface, mineral apposition rate, and bone formation rate were increased compared with 18-month-old rats. A trend toward decreased trabecular number and trabecular thickness was also seen in 21-month-old rats relative to 18-month-old rats. In contrast, bone volume of the rats treated with 0.1 or 0.2  $\mu$ g/kg/d of alfacalcidol was increased by 31% or 45%, respectively, compared with that of vehicle-treated rats. Trabecular number and trabecular thickness were significantly increased whereas trabecular separation was significantly decreased in the rats treated with alfacalcidol compared with baseline and vehicle-treated controls. When comparing the two alfacalcidol-treated groups, the rats treated with the higher dose of alfacalcidol had higher values for bone volume and trabecular thickness but lower values for trabecular separation. Osteoclast surface and osteoclast number were significantly decreased after alfacalcidol treatments relative to vehicle treatments with the osteoclast number decreasing below baseline level. Both mineralizing surface and bone formation rate in the rats treated with alfacalcidol were significantly lower than those of rats treated with vehicle but higher than those of baseline controls. The mineral apposition rate for the rats treated with alfacalcidol at both doses was not different from those treated with vehicle but it was significantly higher for the rats treated with the higher dose of alfacalcidol compared with baseline controls.

**Tibial diaphyses.** Bone histomorphometric data from tibial diaphyses are listed in Table 3 and shown in Figure 2. When compared with the 18-month-old rats, the 21-month-

Variables	Group			
	Baseline	Vehicle	1 $\alpha$ -D <sub>3</sub> (0.1 $\mu$ g)	1 $\alpha$ -D <sub>3</sub> (0.2 $\mu$ g)
Maximum Load (N)	258 $\pm$ 14	224 $\pm$ 12	296 $\pm$ 20 <sup>b</sup>	321 $\pm$ 19 <sup>ab</sup>
Stiffness (N/mm)	1385 $\pm$ 204	1299 $\pm$ 132	1313 $\pm$ 126	1501 $\pm$ 134
Energy Absorbed (mJ)	40.8 $\pm$ 4.1	31.2 $\pm$ 2.9	50.6 $\pm$ 4.0 <sup>b</sup>	52.2 $\pm$ 5.2 <sup>b</sup>
Ultimate Strength (N/mm <sup>2</sup> )	20.4 $\pm$ 1.5	17.9 $\pm$ 0.7	23.4 $\pm$ 1.4 <sup>b</sup>	27.6 $\pm$ 2.0 <sup>ab</sup>
Elastic Modulus (MPa)	456.8 $\pm$ 78.7	427.6 $\pm$ 42.0	452.3 $\pm$ 40.3	625.6 $\pm$ 85.3 <sup>ab</sup>
Toughness (mJ/mm <sup>2</sup> )	0.77 $\pm$ 0.07	0.61 $\pm$ 0.06	0.92 $\pm$ 0.09 <sup>b</sup>	0.94 $\pm$ 0.08 <sup>bc</sup>

Data are expressed as mean  $\pm$  SEM. Baseline: rats euthanized at the beginning of treatments; Vehicle: vehicle-treated controls; 1 $\alpha$ -D<sub>3</sub> (0.1  $\mu$ g): rats treated with 0.1  $\mu$ g/kg/d of alfacalcidol; 1 $\alpha$ -D<sub>3</sub> (0.2  $\mu$ g): rats treated with 0.2  $\mu$ g/kg/d of alfacalcidol. <sup>a</sup>  $p < 0.05$  vs. baseline group; <sup>b</sup>  $p < 0.05$  vs. vehicle-treated controls; <sup>c</sup>  $p < 0.05$  vs. rats treated with 0.1 mg/kg/d of alfacalcidol.

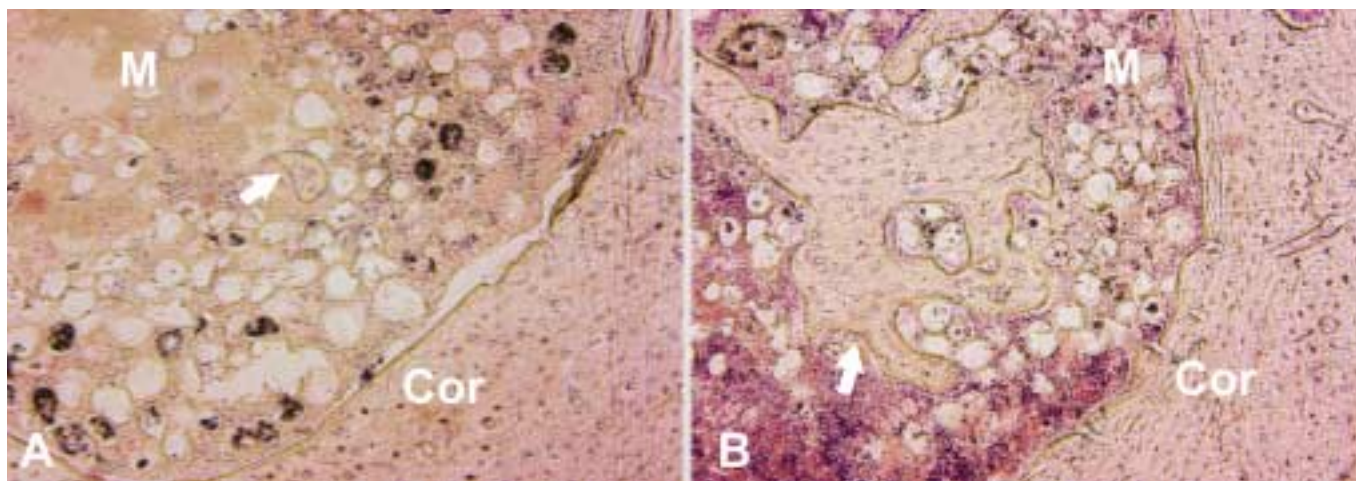
**Table 4.** Biomechanical variables assessed by compression test in lumbar vertebrae (LV<sub>4</sub>).

old rats had a significant increase in percent marrow area, endocortical eroded surface, endocortical mineralizing surface, endocortical mineral apposition rate, and endocortical bone formation rate and a significant decrease in percent cortical area, periosteal mineralizing surface, periosteal mineral apposition rate, and periosteal bone formation rate. The mean values of total tissue area and cortical width in the 21-month-old rats were not significantly different from those of 18-month-old rats. In contrast, the rats treated with alfacalcidol at both doses exhibited significantly increased total tissue area, percent cortical area, cortical width and decreased percent marrow area compared with those treated with vehicle. There was no trabecular bone observed in the marrow cavity of baseline rats. Four out of ten of vehicle-treated rats presented one or two miniscule trabecular spicules, which contributed to the average of 1.5- $\mu$ m<sup>2</sup> marrow trabecular bone area. However, trabecular bone was seen in the marrow cavity of eight out of ten rats treated with 0.1  $\mu$ g/kg/d of alfacalcidol and the average marrow trabecular bone area was increased to 39- $\mu$ m<sup>2</sup>. All rats treated with 0.2  $\mu$ g/kg/d of alfacalcidol had significant amount of trabecular bone in their marrow cavity (Figure 3), which had an average area at 58- $\mu$ m<sup>2</sup>. Endocortical eroded surface of the rats treated with alfacalcidol was significantly decreased relative to those of both baseline and vehicle-treated rats. Interestingly, the mean values for the endocortical bone formation parameters including endocortical mineralizing surface, endocortical mineral apposition rate (except higher dose) and endocortical bone formation rate were significantly lower whereas the mean values for the periosteal bone formation parameters including periosteal mineralizing surface, periosteal mineral apposition rate, and periosteal bone formation rate were significantly higher in the rats treated with alfacalcidol than those treated with vehicle. The periosteal mineral apposition rate and periosteal bone formation rate for the rats treated with higher dose of alfacalcidol were significantly higher than

that those treated with lower dose of the compound. The mean value of periosteal bone formation rate for the rats treated with higher dose of alfacalcidol was also significantly greater than that of baseline controls.

**Bone biomechanical strength.** Mechanical properties of LV<sub>4</sub> as determined by compression testing are shown in Table 4. A trend toward decreased maximum load (-13%), stiffness (-6%), energy (-23%), ultimate strength (-12%), elastic modulus (-6%), and toughness (-21%) was observed in the rats at 21 months of age compared with the rats at 18 months of age. However, the maximum load of LV<sub>4</sub> in the rats treated with 0.1 or 0.2  $\mu$ g/kg/d of alfacalcidol was significantly increased by 32% or 43%, respectively, compared with vehicle-treated rats. The mean value of stiffness was not significantly changed after treatment with either vehicle or alfacalcidol. Energy absorption, ultimate strength, toughness, and elastic modulus were significantly increased by alfacalcidol treatments compared with vehicle treatments. The mean values for maximum load, ultimate strength and toughness in the rats treated with the higher dose of alfacalcidol were also significantly higher than those of baseline controls.

**"Bone Buds".** An unusual pattern of bone formation on endosteal bone surfaces (trabecular and endocortical surfaces) was observed in the rats treated with alfacalcidol (Figure 4). In cancellous bone, an atypical bone formation pattern was observed, characterized by small, focal packets of newly formed bone on trabecular bone surfaces. This gave the microscopic appearance of the formation of "bone buds" emanating from trabecular surfaces. The "bone buds" appeared to be randomly formed along the bone surfaces. Also they were mineralized and demonstrated significant fluorochrome label indicating recent mineralization. Under polarized light, it was observed that the lamellae of the "bone bud" did not run uniformly parallel to those of the trabecular plate to which they are attached. Arrest lines were seen in most of the "bone buds", indicating that the formation of "bone buds" was initiated



**Figure 3.** Microscopic observation of the spicules produced by alfacalcidol treatments in the marrow cavity of tibial diaphysis. Note a very small spicule (arrow) in rat treated with vehicle (A) and two larger size spicules (arrow) in the rats treated with higher dose of alfacalcidol (B). Villanueva bone stained. M: marrow; Cor: cortex. Original magnification: X250.

ed on a quiescent surface. Some "bone buds" had a single arrest line at the border where they connected with the attached trabecular plate. Some "bone buds" had several arrest lines independent of their size. Similar "bone buds" were observed on the endocortical surface. These "bone buds" were observed in the rats treated with higher and lower doses of alfacalcidol with more pronounced in the rats treated with higher dose of the compound. No evidence of osteomalacia was observed in these animals treated with alfacalcidol.

## Discussion

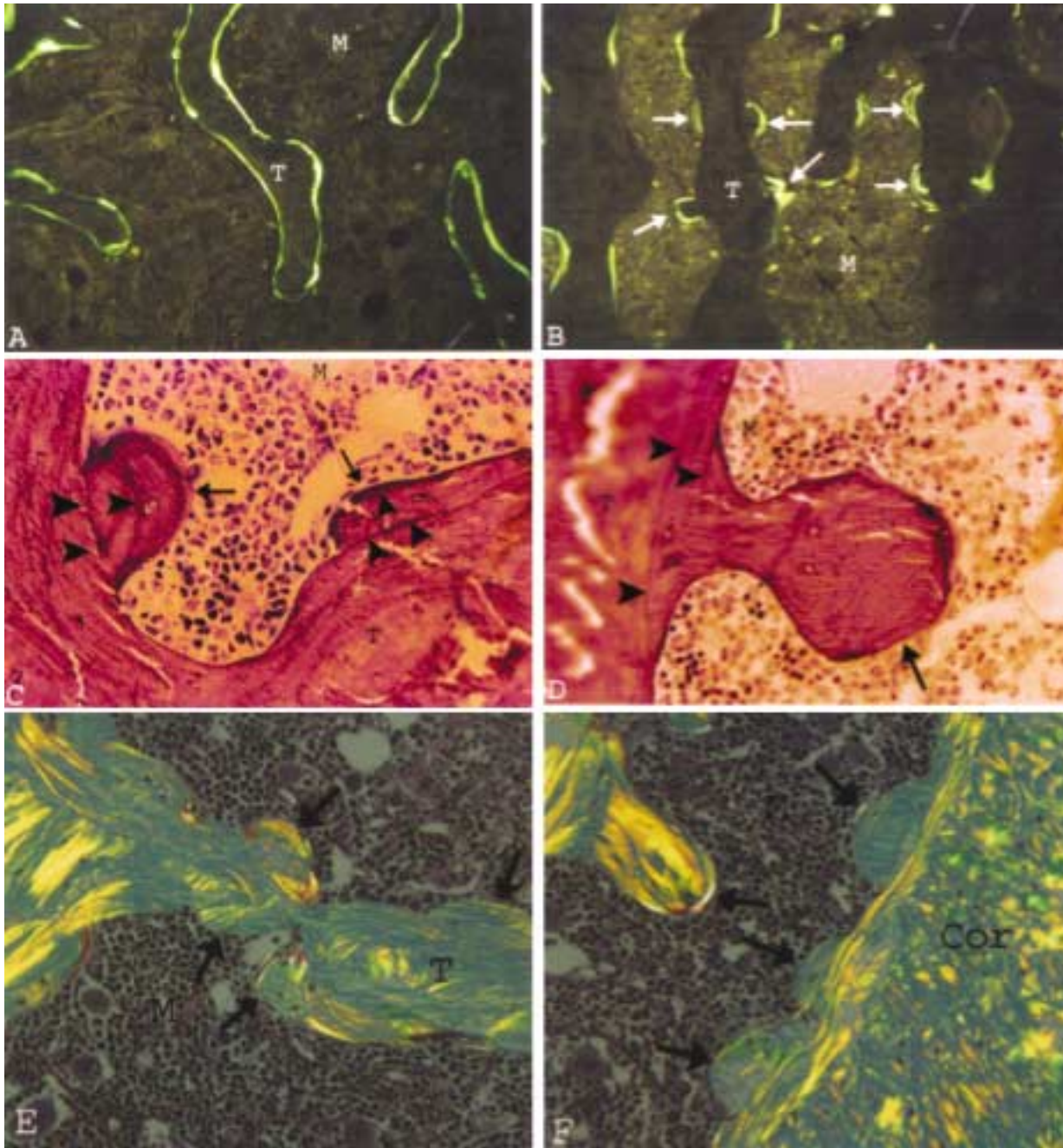
The current study demonstrated that alfacalcidol increased both cancellous and cortical bone mass as well as bone strength, resulting in the prevention of age-related bone loss in aged male rats. The loss of vertebral cancellous bone seen in the rats at 21 months of age relative to those at 18 months of age was associated with increased bone turnover as evident by increased osteoclast surface, mineralizing surface, mineral apposition rate, and bone formation rate in these animals. Similar findings were seen at the proximal tibial metaphyses of the same rats in this study (data not shown). In cortical bone of tibial diaphyses, high bone turnover was evident on the endocortical surface whereas decreased bone formation was seen on the periosteal surface, resulting in a decrease in percent cortical bone area and an increase in percent marrow area. These changes in the 21-month-old male rats were similar to those reported in the androgen deficient rats induced by orchidectomy<sup>49-51</sup>. Therefore, the significantly decreased testosterone level toward a hypogonadal state observed in this study is a likely cause of bone loss in these 21 month-old male rats.

Consistent with previous findings in relatively young rats treated with active vitamin D metabolites<sup>33-35,37-40,52</sup>, our data showed that alfacalcidol dose-dependently increased cancellous bone mass and improved trabecular structure resulting

in an increase of bone strength in the lumbar vertebral bodies of aged male rats. In addition, alfacalcidol increased cortical bone mass by inhibiting the increased bone resorption and turnover on the endocortical surface and by stimulating bone formation on the periosteal surface in aged rats. These effects of alfacalcidol on bone completely prevented the age-related cancellous bone loss and cortical bone changes and added additional bone to the skeleton of aged rats. In concert with previous studies in younger rats, the current study demonstrates that the skeletal effects of alfacalcidol are independent of age in rats.

The suppressed bone resorption on the trabecular and endocortical surfaces by alfacalcidol treatment in aged male rats in the current study is consistent with the previous findings in relatively young rats<sup>33-35,37,39,40,52</sup>. Similar effects have also been documented in OVX dogs<sup>53,54</sup> and postmenopausal women treated with calcitriol<sup>5</sup>. The inhibitory effect of alfacalcidol on bone resorption was accompanied with a dose-dependent increase in serum calcium levels in aged rats. The observed elevation of serum calcium is probably due to increased intestinal absorption of calcium by alfacalcidol treatments. Although serum PTH levels were not determined in this study, previous studies have clearly shown decreased serum PTH levels after alfacalcidol treatments in rats<sup>34,40</sup>. Since PTH is an important physiological regulator of bone resorption and bone turnover, the suppression of bone resorption by alfacalcidol in the current study is considered to be largely a consequence of diminished PTH activity.

The effects of alfacalcidol treatment on bone formation in aged male rats were varied on different bone surfaces. In cancellous bone of lumbar vertebra, although bone formation rate in aged rats treated with alfacalcidol was lower than that of age-matched controls, but trabecular bone volume of alfacalcidol-treated rats was significantly increased above baseline controls. These data indicated that alfacalcidol treatment, if not increased, at least maintained the overall bone



**Figure 4.** Microscopic observation of the "bone buds" produced by alfacalcidol treatments. Lumbar vertebral cancellous bone tissue from a vehicle-treated rat (A) and alfacalcidol treated rats (B-F). Note "bone buds" (arrows) appear on the trabecular and endocortical surfaces in the rat treated with higher dose of alfacalcidol (B-F). The "bone buds" have significant fluorochrome label (B) and possess single or multiple smooth cement lines (arrow heads, C and D). The lamellae of "bone buds" do not run parallel to those of the trabecular plate or cortex to which they are attached (E and F, viewed under polarized light). Unstained (A and B), toluidine blue surface stain (C and D), and modified masson's trichrom stain (E and F). T: trabecular bone; M: marrow; Cor: cortex. Original magnification: X250.

formation in the aged male rats. In other words, during the course of the alfacalcidol treatment period, the combination of maintained bone formation and decreased bone resorption on cancellous bone surfaces of aged rats produced a positive bone balance between bone formation and resorption resulting in thickening of trabecular bone and a net bone gain

in these aged animals. In cortical bone, our data showed that alfacalcidol treatment prevented the increased bone resorption and maintained bone formation on the endocortical bone surface in aged rats with decreased testosterone levels. These combination effects resulted in a net bone gain on this bone surface and accounted for a decrease in marrow cavity



area. On the periosteal surface, alfacalcidol not only prevented the decreased bone formation but also maintained (at lower dose) or even increased (at higher dose) bone formation in aged rats. Interestingly, marrow trabecular bone of tibial diaphyses was significantly increased in aged rats after 12 weeks of alfacalcidol treatment in the current study. In considering the lack of trabecular bone spicules in the baseline rats, this finding may suggest that alfacalcidol treatments also stimulated bone formation without a pre-existing bone surface and created new trabecular spicules in marrow cavity in aged rats. The disparity between the existence of a few spicules of bone in the 21 month-old vehicle group as compared to the 18 month-old baseline group can not be explained. It is noted that this is a rare occurrence and only a very small amount of bone is observed. Since one or two very small trabecular spicules were observed at the tibial diaphyses in four out of ten vehicle-treated rats, we cannot rule out the possibility that alfacalcidol treatment enlarged previously existing spicules within bone marrow.

Although alfacalcidol treatment at the doses used in the current study dose-dependently increased bone on all bone surfaces in aged male rats, its action on different surfaces was varied. On the trabecular or endocortical surfaces, where bone resorption and turnover were increased, alfacalcidol treatment inhibited osteoclast bone resorption and decreased bone turnover, and at the same time it stimulated focal bone formation and formed "bone buds" (discussed next). On the periosteal surfaces, where bone formation was decreased, alfacalcidol treatment stimulated bone formation. However, whether the effects of alfacalcidol and other active vitamin D analogs on bone formation are direct or indirect due to the increased intestinal calcium absorption remains unclear. The data from studies with Ro-26-9228, a vitamin D analog reported to be selective in osteoblast cells compared with intestinal cells support a direct effect of active vitamin D on bone formation. This analog up-regulates osteoblast-specific gene products such as osteocalcin and osteopontin as well as TGF- $\beta$ 1 and - $\beta$ 2 mRNA<sup>55</sup>. It also increases osteoblast number and cancellous bone mass at a noncalcemic dose in OVX rats. Another vitamin D analog, ED-71, provided a similar profile<sup>56,57</sup> and has been shown to increase lumbar vertebral bone mineral density in osteoporotic patients<sup>58</sup>. However, highly selective or bone specific analogs with significantly reduced or eliminated hypercalcemic effects are needed for fully understanding the direct action of active vitamin D on bone.

Another interesting finding of this study was an atypical pattern of bone formation, "bone buds," observed in the rats treated with alfacalcidol for 3 months. This finding has not been previously reported although similar experiments have been carried out in relatively younger rats, which were treated with the same dose and duration of alfacalcidol<sup>37</sup>. However, a similar observation was reported by Erben et al. in adult rats having received pharmacological doses of calcitriol<sup>59,60</sup>. Based on their microanatomical feature, the "bone buds" are likely a result of the accumulation of micro packet bone formation on bone surface stimulated by alfacalcidol treatment mainly through a minimodeling processing, where

bone formation occurs on quiescent bone surfaces without preceding osteoclast bone resorption<sup>61</sup>. Evidence of minimodeling was found in tibial and vertebral cancellous bone in rats<sup>42</sup> and more importantly, it has also recently been reported in the transiliac biopsy specimens from the patients who underwent total hip arthroplasty<sup>62</sup>. The appearance of the focally formed bone at the minimodeling site in these human bone specimens is similar to some of the "bone buds" observed in the rats treated with alfacalcidol in the current study. Another interesting feature of the "bone buds" is the successive layers of arrest lines. Some "bone buds" have several layer of arrest lines suggesting that they have formed through multiple formation periods or cycles. On the other hand, some "bone buds" have only a single arrest line that is located at the base of the "bone buds" suggesting that they may have formed by a single continuous formation period. The amount of bone formed in the "bone buds" within single arrest line suggests that alfacalcidol treatment may have augmented the ability of osteoblast to synthesize and mineralize matrix. Changes in the microenvironment in the marrow cavity, significantly increased serum calcium levels, and the duration of the treatment may contribute to the initiation of this microscopic pattern of locally destined bone formation.

In summary, alfacalcidol treatment increased cancellous and cortical bone mass and improved bone strength in aged male rats, resulting in the prevention of age-related bone loss. A unique pattern of bone formation observed in this study may be a result of minimodeling based bone formation stimulated by alfacalcidol treatment.

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