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Original Article

Hylonome

Greater efficacy of alfacalcidol in the red than in the yellow marrow skeletal sites in adult female rats

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Abstract

The present study compared the bone anabolic effects of graded doses of alfacalcidol in proximal femurs (hematopoietic, red marrow skeletal site) and distal tibiae (fatty, yellow marrow skeletal site). One group of 8.5-month-old female Sprague-Dawley rats were killed at baseline and 4 groups were treated 5days on/2days off/week for 12 weeks with 0, 0.025, 0.05 and 0.1 μg alfacalcidol/kg by oral gavage. The proximal femur, bone site with hematopoietic marrow, as well as the distal tibia bone site with fatty marrow, were processed undecalcified for quantitative bone histomorphometry. In the red marrow site of the proximal femoral metaphysis (PFM), 0.1 μg alfacalcidol/kg induced increased cancellous bone mass, improved architecture (decreased trabecular separation, increased connectivity), and stimulated local bone formation of bone "boutons" (localized bone formation) on trabecular surfaces. There was an imbalance in bone resorption and formation, in which the magnitude of depressed bone resorption greater than depressed bone formation resulted in a positive bone balance. In addition, bone "bouton" formation contributed to an increase in bone mass. In contrast, the yellow marrow site of the distal tibial metaphysis (DTM), the 0.1 μg alfacalcidol/kg dose induced a non-significant increased cancellous bone mass. The treatment decreased bone resorption equal to the magnitude of decreased bone formation. No bone "bouton" formation was observed. These findings indicate that the highest dose of 0.1 μg alfacalcidol/kg for 12 weeks increased bone mass (anabolic effect) at the skeletal site with hematopoietic marrow of the proximal femoral metaphysis, but the increased bone mass was greatly attenuated at the fatty marrow site of the distal tibial metaphysis. In addition, the magnitude of the bone gain induced by alfacalcidol treatment in red marrow cancellous bone sites of the proximal femoral metaphysis was half that of the lumbar vertebral body. The latter data were from a previous report from the same animal and protocol. These findings indicated that alfacalcidol as an osteoporosis therapy is less efficacious as a positive bone balance agent that increased trabecular bone mass in a non-vertebral skeletal site where bone marrow is less hematopoietic.

Keywords: Alfacalcidol, Proximal Femur, Distal Tibia, Hematopoietic (Red) Marrow Site, Fatty (Yellow) Marrow Site, Histomorphometry

Introduction

Pre-clinical studies in rodent animal models have shown that the effects of current or potential osteoporosis drugs on fatty marrow skeletal sites differ. Both parathyroid hormone (PTH) and prostaglandin E2 (PGE_2) stimulate cancellous bone formation at skeletal sites regardless of marrow composition[1-4]. In contrast, basic fibroblast growth factor treatment lacked a significant bone anabolic effect at the fatty distal tibial metaphyses (DTM) in ovariectomized rats[5]. It is not known whether alfacalcidol, a vitamin D analog, would be beneficial in skeletal sites regardless of marrow composition.

Recently, we reported the positive bone balance response of alfacalcidol in proximal tibial metaphysis, lumbar vertebral body and tibial shaft in intact female rats, sites with hematopoietic (red) marrow sites[6,7]. The effect on fatty (yellow) marrow skeletal sites was not studied. Because some common fracture sites in osteoporotic patients, as the distal radius and ulna (non-vertebral sites) are composed of fatty (yellow) marrow[8], the responses of fatty marrow sites to osteoporosis drugs are needed. A fatty marrow site in the 8.5-month-old female rat is

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Age	8.5 mo.	11.5 mo.	
Experimental period			**12 weeks**
1. Baseline	6		N/A
2. Aging control		6	cottonseed oil
3. Alfacalcidol 0.025		7	0.025 μg/kg
4. Alfacalcidol 0.05		7	0.05 μg/kg
5. Alfacalcidol 0.1		7	0.1 μg/kg
Graded doses of alfacalcidol, oral gavage, 5 days/week for 12 weeks.			

Table 1. Experimental design.

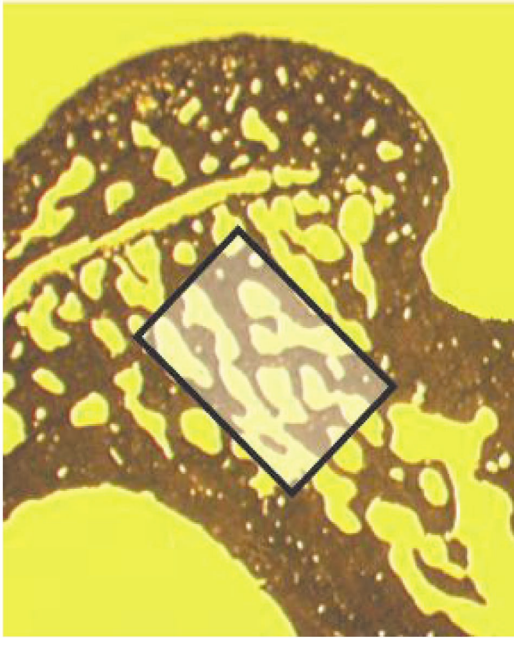

Figure 1. Proximal femur metaphysis (PFM), showing sample area within the femoral neck for measurement of cancellous bone. It begins 1 mm distal to the growth plate and extends nearly to the junction of the femoral neck and greater trochanter. Von Kossa staining; 1X magnification.

the distal tibia. The distal tibia fuses its epiphysis at 3 months and is predominately a fatty marrow site with a cancellous bone histomorphometric profile similar to that seen in human iliac crest biopsy sites[1,9]. In addition, unknown is how anabolically efficacious alfacalcidol treatment is in the proximal femoral metaphysis compared to the proximal tibial metaphyses and lumbar vertebral bodies, both red marrow bone sites. Therefore, the current report determined by histomorphometry the anti-catabolic and anabolic effects of alfacalcidol to induce bone gain by: (1) cancellous bone at hematopoietic (red) proximal femoral metaphysis and fatty (yellow) distal tibial metaphysis sites; and (2) cancellous bone in two different hemotopoietic (red) marrow sites (proximal femoral metaphysis and lumbar vertebral body) from the same animals[6].

Materials and methods

Experimental protocol

Thirty-three 3-month-old virgin female Sprague-Dawley rats (Charles River Laboratories, Portage, MI) were maintained on a 12:12 h light/dark cycle at 72°F. The rats were allowed free access to water and a pelleted commercial natural diet (Teklad Rodent Laboratory Chow 8640 Harlan Teklad, Madison, WI) containing 1.13% calcium, 0.94% phosphorus and 2.99 IU/g of vitamin D_3. The rats were weighed weekly throughout the study. The animal protocol was approved by the University of Utah Institutional Animal Care and Use Committee to ensure compliance with NIH guidelines.

At 8.5 months of age, the rats were divided into 5 groups (6 or 7 rats per group) (Table 1). One group was killed on day 1 as baseline controls, and the rest of the rats received vehicle (cottonseed oil, Sigma-Aldrich, Inc., St. Louis, MO) or alfacalcidol (Calbiochem, La Jolla, CA) 5 days on/2 days off/week by oral gavage. Alfacalcidol stock solution was made by dissolving the compound 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 cottonseed oil to the concentration of 0.025, 0.05, and 0.1 μg/ml for the 0.025, 0.05, and 0.1 μg/kg groups, respectively. The selection of dose was based on previous studies in OVX'd rats[10-21]. The lowest efficacious dose was 0.05. Therefore, we decided on the 3 dose levels with one lower than found in the literature in case the 2 sites chosen were more reactive in the intact female rat. All the rats (including baseline) received sc injections with Calcein (10 mg/kg; Sigma Chemical Co., St. Louis, MO) on days 14, 13, and 4, 3 before sacrifice. One day after the final treatment, animals were anesthetized with Avertin (0.5 g/kg body weight, Sigma-Aldrich Inc., St. Louis, MO) and euthanized via exsanguinations to permit collection of a blood sample by open-chest cardiac puncture. The proximal femurs and distal tibiae were harvested for histomorphometry.

Serum biochemistry

Blood was obtained from open-chest cardiac puncture; the serum samples were from centrifuged blood. The samples were stored at -80°C until the assay. Serum calcium (Ca) and inorganic phosphorus (Pi) concentrations were measured with a Cobas Fara 2 analyzer (Roche Diagnostic System, Hoffman-La Roche Inc., Indianapolis, IN) at Pfizer Global Research and Development Inc., Groton, CT, USA.

Bone histomorphometry

The left proximal femurs were placed in 10% phosphate-buffered formalin for 24 hours for tissue fixation. Both prox-

	Baseline	Aging Control	0.025	0.05	0.1
Serum calcium (mg/dl)	11.20±0.30	10.68±0.21[a] (-5)	10.76±0.41 (-4; 1)	10.94±0.39 (-2; 2)	11.23±0.44[b] (0; 5)
Serum inorganic phosphorus (mg/dl)	5.33±0.33	4.83±0.87 (-9)	6.06±0.94[b] (14; 25)	6.26±0.83[ab] (17; 29)	6.80±0.89[ab] (28; 41)

Mean±SD; (%change from Baseline; or/and %change from Aging control), [a]$p<0.05$ vs. Baseline; [b]$p<0.05$ vs. Aging control.

Table 2. Serum biochemistry.

imal femurs and distal tibiae were stained en bloc with Villanueva Bone Stain (Arizona Histology & Histomorphometry Center, Phoenix, AZ, USA), dehydrated in graded concentrations of alcohol, defatted in acetone, and embedded in methyl methacrylate monomer (Fisher Scientific, Fairlawn, NJ, USA). The proximal femur sectioning began at approximately one-third the depth of the femoral neck. These longitudinal sections of the proximal femur (PFM) were cut at 4 and 8 μm thickness with a Leica RM 2165 microtome (Leica, Nussloch, Germany). One set of 4 μm-thick sections were stained with 5% Von Kossa, one set of 4 μm-thick sections were stained with 0.1% Toluidine Blue Stain and the 8 μm-thick sections remained unstained[22,23]. The distal tibial metaphysis (DTM) cross-sections were taken approximately 4mm from the distal end and cut at a 230 μm thickness using a Low Speed Diamond Saw (Isomet, Buehler, Lake Bluff, IL, USA), and then hand ground to 20 μm for histomorphometric analyses[24].

All bone measurements were performed using a semi-automatic Image Analysis System (OsteoMeasure, OsteoMetrics Inc. Decatur, GA, USA). Cancellous bone measurements were performed in the proximal femur in an area beginning 1mm distal to the growth plate-metaphyseal junction and extending distally to the junction of the femoral neck and greater trochanter[22,23] (Figure 1). In the cross sections of distal tibiae, cancellous and cortical bone from the mid-distal tibial metaphysis were measured.

Measurement parameters of cancellous bone included total tissue area (T.Ar), trabecular bone area (B.Ar), trabecular bone perimeter (B.Pm), numbers of nodes (N#), cortical bone to free end (CTF), node to free end (NTF), free end to free end (FTF), node to node (NTN), cortical bone to node (CTN), single- (sL.Pm) and double-labeled perimeter (dL.Pm), interlabel width (Ir.L.Wi), osteoid perimeter (O.Pm), eroded perimeter (Er.Pm [DTM]) or osteoblast perimeter (Ob.Pm [PFM]), osteoclast perimeter (Oc.Pm [PFM]) and osteoclast number (Oc.N [PFM]). These indices were used to calculate percent trabecular bone area (%B.Ar), trabecular width (Tb.Wi), trabecular number (Tb.N), trabecular separation (Tb.Sp), numbers of free ends; the ratio of nodes number/free ends number (N/F) and density of connectivity variables based on B.Ar, and T.Ar, the mineralized perimeter (%L.Pm), mineral apposition rate (MAR), percent osteoid perimeter (%O.Pm), percent eroded perimeter (%Er.Pm [DTM]) and bone formation rate per unit of bone area, tissue area and bone surface (BFR/B.Ar, BFR/T.Ar, BFR/BS), percent osteoblast perimeter (%Ob.Pm [PFM]), percent osteoclast perimeter (%Oc.Pm [PFM]), osteoclast number/trabecular surface (Oc.N/B.Pm [PFM])[22,23,25-28]. Mineral appositional rate (MAR) was assigned the value 0.1 μm/d if no double labels were observed[29]. The estimate index of tissue level bone balance (%Ob.Pm/%Oc.Pm or %L.Pm/%Er.Pm) and the index of BMU bone balance (%Er.Pm-%L.Pm or %Oc.Pm–%Ob.Pm) were determined compared to aging controls[30-32].

The histomorphometry of cancellous bone in thin longitudinal sections of PFM was compared to that in thick cross-sectional DTM. The approach was valid in that a recent study showed data derived from these two types of samples were similar[33].

Statistical analysis

Data were presented as a group means±standard deviation (SD). Statistics were calculated by an Ultimate Integrated Data Analysis and Presentation System (StatView 5.0.1, SAS Institute Inc., Cary, NC, USA). Across group comparisons were made with a parametric analysis of variance (ANOVA), followed by Fisher's protested least significant difference test (PLSD). Differences were considered statistically significant at $p<0.05$.

Results

Body, muscle weights and serum biochemistry

The body and muscle weights did not differ among groups (data not shown). Because the baseline serum calcium level was higher than normal for some unclear reason, we restricted our comparison to aging control levels. Significant increased serum calcium (+5%) compared to aging control was limited to the 0.1 μg/kg alfacalcidol dose, but the increase was no more than that seen in the baseline. Serum inorganic phosphorus (Pi) increased in a dose-response manner: 0.025, 0.05, 0.1 μg/kg alfacalcidol increased +25%, +29% and +41% vs. aging control, respectively (Table 2).

	Baseline	Aging Control	0.025	0.05	0.1
Animals per group (n, #)	6	6	7	7	7
Trabecular bone area (%B.Ar, %)	51.73±3.32	51.05±3.05 (-1)	53.75±1.05 (4; 5)	51.47±8.25 (-1; 1)	61.61±6.41[ab] (19; 21)
Trabecular width (Tb.Wi, mm)	122.58±7.34	120.48±20.60 (-2)	130.89±13.10 (7; 9)	112.52±22.82 (-8; -7)	141.38±28.21 (15; 17)
Trabecular number (Tb.N, #)	4.23±0.40	4.33±0.72 (2)	4.14±0.36 (-2; -5)	4.62±0.45 (9; 6)	4.44±0.54 (5; 3)
Trabecular separation (Tb.Sp, mm)	115.21±16.80	115.48±20.17 (0)	112.48±9.28 (-2; -3)	105.83±19.74 (-8; -8)	87.06±15.13[ab] (-24; -25)
Ratio of nodes number/ Free ends number (N/F)	3.40±0.94	3.26±0.58 (-4)	4.74±1.94 (39, 45)	5.41±3.28 (59, 66)	6.89±2.52[ab] (103, 111)
Percent osteoid perimeter (%O.Pm, %)	6.00±2.09	6.11±3.27 (2)	4.06±1.32 (-32; -34)	3.86±1.85 (-36; -37)	3.62±2.09 (-40; -41)
Mineralizing surface (MS/BS, %)	22.07±2.74	21.75±3.29 (-1)	12.24±4.87[ab] (-45; -44)	12.46±4.37[ab] (-44; -43)	10.64±5.35[ab] (-52; -51)
Mineral apposition rate (MAR, mm/day)	0.97±0.10	0.96±0.18 (-1)	0.93±0.10 (-4; -3)	1.00±0.17 (2; 3)	1.02±0.25 (5; 6)
Bone formation rate (BFR/BS, mm^3/mm^2/day x 100)	21.49±3.79	20.66±3.51 (-4)	11.32±4.46[ab] (-47; -45)	12.34±4.89[ab] (-43; -40)	12.05±8.24[ab] (-44; -42)
Bone formation rate (BFR/B.Ar, %/year)	106.86±18.02	106.07±19.47 (-1)	51.91±17.18[ab] (-51; -51)	69.29±30.75[ab] (-35; -35)	48.05±25.50[ab] (-55; -55)
Bone formation rate (BFR/T.Ar, %/year)	55.32±10.07	54.23±10.91 (-2)	27.98±9.61[ab] (-49; -48)	34.46±13.33[ab] (-38; -36)	30.47±18.50[ab] (-45; -44)
Percent osteoblast perimeter (%Ob.Pm, %)	16.59±3.16	20.35±3.55 (23)	16.57±5.36 (0; -19)	14.24±5.09[b] (-14; -30)	13.45±4.25[b] (-19; -34)
Percent osteoclast perimeter (%Oc.Pm, %)	2.33±0.49	2.89±0.43 (24)	2.38±1.11 (2; -18)	1.67±0.44[ab] (-28; -42)	1.51±0.48[ab] (-35; -48)
Osteoclast number/bone surface (Oc.N/BS, #/mm)	1.42±0.26	1.72±0.31 (21)	1.41±0.69 (-1; -18)	1.03±0.25[ab] (-27; -40)	0.90±0.33[ab] (-37; -48)
Bone balance (%Ob.Pm / %Oc.Pm)	7.16±0.77	7.22±1.85 (1)	7.99±3.52 (12; 11)	8.61±2.45 (20; 19)	8.92±3.24 (25; 24)
BMU balance (%Oc.Pm - %Ob.Pm)			-1	10	14

Mean ± SD; (%change from Baseline; or/and %change from Aging control), [a]$p<0.05$ vs. Baseline; [b]$p<0.05$ vs. Aging control.

Table 3. Selective trabecular bone histomorphometric parameters of proximal femoral metaphysis.

Histomorphometry of cancellous bone of proximal femur metaphysis (PFM)

There were no significant age-related static and dynamic histomorphometry changes.

The 0.025 and 0.05 μg alfacalcidol/kg treatments had no effect on bone mass, architecture and bone balance. It decreased the magnitude of bone resorption (%Oc.Pm: -18 and -42% with 0.025 and 0.05 μg/kg doses) and formation (%Ob.Pm: -19 and -30% with 0.025 and 0.05 μg/kg) were similar [i.e., the coupling of bone formation to resorption phases was in balance (equilibrium)].

Only the 0.1 μg/kg alfacalcidol treatment significantly increased cancellous bone mass (%B.Ar, +21%) and improved architecture (decreased trabecular separation: Tb.Sp, -25%; increased connectivity: +111%), caused by the decreased bone resorption (%Oc.Pm, -48%; Oc.N/BS, -48%), bone turnover (BFR/B.Ar, -55%) and bone formation (%Ob.Pm, -34%; BFR/BS, -42%). The magnitude of the depressed resorption index (%Oc.Pm, -48%) was greater than the formation (%Ob.Pm, -34%) signifying more depression of bone resorption than bone formation, which signified a stimulation. This imbalance caused a non-significant increase in estimated positive tissue level bone balance of +24%, and BMU bone balance of +14% compared to aging controls (Table 3).

"Boutons" formation and "Non-Boutons" formation surfaces in cancellous bone of PFM

The local bone formation sites ("boutons")[34,35] were observed on the trabecular surface treated with alfacalcidol

	"Bouton" Bone Surface					Non "Bouton" Bone Surface		
	Frequency (B/Animal)	**B.N (#)**	**%L.Pm**	**MAR**	**BFR/BS**	**%L.Pm**	**MAR**	**BFR/BS**
Baseline Control	0/6	1.00	1.00	1.00	1.00	22.07±2.74	0.97±0.10	21.49±3.79
Aging Control	0/6	1.00 (0)	1.00 (0)	1.00 (0)	1.00 (0)	21.75±3.29 (-1)	0.96±0.18 (-1)	20.66±3.51 (-4)
Alfacalcidol 0.005 μg/kg	0/7	1.00 (0; 0)	1.00 (0; 0)	1.00 (0; 0)	1.00 (0; 0)	23.99±9.87 (9; 10)	1.01±0.19 (4; 5)	24.86±11.45 (16; 20)
Alfacalcidol 0.025 μg/kg	1/6	2.00 (100; 100)	61.07 (6007; 6007)	1.71 (+71; +71)	43.65 (+4265; 4265)	12.24±4.87 (-45; -44)	0.93±0.10 (-4; -3)	11.32±4.46 (-47; -45)
Alfacalcidol 0.05 μg/kg	3/7	2.67±1.15 (167; 167)	61.87±7.60 (6087; 6087)	1.90±0.28 (+90; +90)	57.07±22.26 (5607; 5607)	12.46±4.37 (-44; -43)	1.00±0.17 (2; 3)	12.34±4.89 (-43; -40)
Alfacalcidol 0.1 μg/kg	7/7	3.14±0.99 (214; 214)	74.34±10.31 (7334; 7334)	1.88±0.19 (+88; +88)	64.63±12.33 (6363; 6363)	10.64±5.35 (-52; -51)	1.02±0.25 (5; 6)	12.05±8.24 (-44; -42)

Mean ± SD; (%change from beginning control; %change from aging control).

Table 4. Comparison of "boutons" bone surface and non "boutons" bone surface.

at all dose levels. The labeled "boutons" area from the last 14 days of 0.025 to 0.1 μg/kg treatment occupied 0.008% to 0.085% of total tissue area. The labeled "boutons" were limited to one animal at 0.025 μg/kg, three animals at 0.05 μg/kg, and every animal in the 0.1 μg/kg dose group. At the highest dose, 0.1 μg/kg, the "boutons" bone surface label perimeters (%L.Pm) and bone surface-based bone formation rate (BFR/BS) were 73 and 64 times more than that of "non-boutons" surfaces, respectively (Table 4).

Histomorphometry of cancellous bone of distal tibial metaphysis (DTM)

There were no significant age-related changes in mass or architecture; however, significant differences were observed in decreased bone turnover (BFR/B.Ar, -43%) and formation (BFR/BS, -49%) along with increased resorption (%Er.Pm, +73%).

A lack of significant change in bone mass was observed at all dose levels. The 0.1 μg/kg alfacalcidol treatment depressed equally the indices of bone formation (MS/BS: -81%; BFR/BS: -83%; BFR/T.Ar: -80%), index of total bone resorption (%Er.Pm: -82%) and bone turnover (BFR/B.Ar: -85%). There was no significant change in bone balance (+7%) nor BMU bone balance of +1% in that decreased bone resorption equaled bone formation (Table 5). In addition, no "boutons" were observed.

Comparison of net changes in select parameters between red PFM and yellow DTM

A comparison of the net change between the 2 sites found the red marrow PFM site increased bone mass, trabecular width and connectivity (Node/Free ratio) of 2, 2.7 and 5.8 fold, respectively, compared to the yellow marrow DTM site. In addition, there was less depression of bone turnover (BFR/B.Ar) and bone formation (BFR/BS and %L.Pm) in the red marrow PFM than in yellow marrow DTM that favored a factor of 14 in BMU bone balance over that in the DTM (Table 6).

Discussion

The major side effect of alfacalcidol or any other vitamin D metabolite has focused on the occurrence of hypercalcemia. The current study reports a mild increase in serum calcium of 5% compared to aged controls with the anabolic 0.1 μg alfacalcidol/kg dose. More surprising was the dose response increase in serum phosphorus of +41% at this dose level (Table 2). These increases may have been contributed to by the high calcium and phosphate levels in the diet and limited to intact female rats. Nevertheless, future studies should be aware of the indisputable pathologic role of hyperphosphatemia raising the risk of cardiovascular death due to abnormal vascular calcification[36-38].

The current study demonstrates that alfacalcidol treatment of cancellous bone in the proximal femoral metaphysis (PFM), a hematopoietic (red) marrow site, increased trabecular bone mass and micro-architecture, reduced trabecular separation and increased connectivity by decreased bone turnover and bone resorption more than decreasing bone formation (depressed resorption and stimulated formation) producing an estimated positive cancellous bone balances compared to controls. In addition, local bony proliferation (boutons) occurred at doses as low as 0.025 μg/kg. In contrast, alfacalcidol-treated DTM, a yellow or fatty marrow site, showed non-significant increases in cancellous bone

	Baseline	Aging Control	0.025	0.05	0.1
Animals per group (n, #)	6	6	7	7	7
Trabecular bone area (%B.Ar, %)	36.97±10.65	35.18±8.60	35.44±9.79 (-5)	31.70±5.64 (-4; 1)	42.02±7.56(14; 19) (-14; -10)
Trabecular width (Tb.Wi, μm)	87.97±14.89	79.78±8.00 (-9)	81.46±17.20 (-7; 2)	78.62±15.01 (-11; -1)	95.44±19.00 (8; 20)
Trabecular number (Tb.N, #)	4.20±0.99	4.41±0.97 (5)	4.34±0.67 (3; -1)	4.10±0.77 (-2; -7)	4.48±0.82 (6; 2)
Trabecular separation (Tb.Sp, μm)	161.13±60.45	156.23±50.53 (-3)	153.68±40.73 (-5; -2)	172.46±40.40 (7; 10)	134.24±34.93 (-17; -14)
Ratio of nodes number/ Free ends number (N/F)	4.49±3.03	3.88±2.32 (-14)	3.96±1.75 (-12, 2)	4.68±2.60 (4, 20)	5.03±3.46 (12, 30)
Percent osteoid perimeter (%O.Pm, %)	5.66±2.76	4.98±1.69 (-12)	5.28±1.51 (-7; 6)	5.59±0.50 (-1; 12)	3.08±1.50 (-46; -38)
Mineralizing surface (MS/BS, %)	28.87±7.64	17.95±9.37 (-38)	13.43±7.97[a] (-53; -25)	14.54±6.34[a] (-50; -19)	3.38±2.78[ab] (-88; -81)
Mineral apposition rate (MAR, μm/day)	0.84±0.11	0.68±0.09 (-20)	0.59±0.11[a] (-30; -12)	0.62±0.27 (-27; -9)	0.59±0.15[a] (-30; -12)
Bone formation rate (BFR/BS, $\mu m^3/\mu m^2$/dayx100)	24.59±7.41	12.55±7.17[a] (-49)	8.38±5.50[a] (-66; -33)	9.87±5.34[a] (-60; -21)	2.18±2.13[ab] (-91; -83)
Bone formation rate (BFR/B.Ar, %/year)	171.79±50.98	97.80±57.27[a] (-43)	65.95±46.83[a] (-62; -33)	84.36±53.89[a] (-51; -14)	14.96±16.78[ab] (-91; -85)
Bone formation rate (BFR/T.Ar, %/year)	64.77±27.32	31.68±15.00[a] (-51)	22.67±15.99[a] (-65; -28)	24.64±13.88[a] (-62; -22)	6.24±7.24[ab] (-90; -80)
Percent eroded perimeter (%Er.Pm, %)	0.43±0.23	0.74±0.23[a] (73)	0.36±0.18 (-17; -52)	0.27±0.10[b] (-36; -63)	0.13±0.05[ab] (-69; -82)
Bone balance (%Ms/BS / %Er.Pm)	99.08±71.08	27.54±16.66[a] (-72)	49.52±42.75 (-50; 80)	62.69±37.39 (-37; 128)	29.36±28.79 (-70; 7)
BMU balance (%Er.Pm - %L.Pm)			27	44	1

Mean ± SD; (%change from Baseline; or/and %change from Aging control), [a]$p<0.05$ vs. Baseline; [b]$p<0.05$ vs. Aging control.

Table 5. Selective trabecular bone histomorphometric parameters of DTM.

mass and connectivity and decreased trabecular separation. The alfacalcidol treatment equally decreased bone resorption, bone turnover and bone formation parameters culminating in equilibrium in bone balances. In addition, no "bouton" formation was observed.

The current study found only the 0.1 μg/kg alfacalcidol dose with its anti-catabolic and anabolic properties significantly enhanced cancellous bone gain and trabecular microstructure in red marrow PFM of adult female rats. The increased cancellous bone mass was the result of positive cancellous bone balance of suppressed resorption greater than depressed formation and the stimulation of "boutons" formation from minimodeling and mixed remodeling/minimodeling formations[34,35,39-41].

The alfacalcidol-induced proximal femoral metaphyseal (PFM) cancellous bone gain mechanisms involved the anti-catabolic and anabolic properties of alfacalcidol previously reported in the LVB and PTM in OVX'd[10-21], intact aged male[35] and female[6,7] rats. It involved positive remodeling/modeling bone balances-induced bone gain and improved connectivity by decreased bone resorption and stimulated formation partially from inhibiting osteoblast apoptosis[42,43]. The positive BMU bone balance occurred from more suppressed bone resorption than bone formation, a functional imbalance in favor of bone formation. Alfacalcidol treatment directly and indirectly decreased osteoclastic activity, bone turnover, activation frequency, BMU resorption cavity depth to maintain or slightly increase bone mass. The stimulated osteoblastogenesis increased mean wall thickness and/or overfilled resorption cavities as mixed remodeling/minimodeling formation sites similar to tissue-level events described recently in publications with parathyroid hormone (PTH) treatment[40,41] to further increase bone mass. The modeling-dependent bone gain and improved architecture involving the stimulation of "boutons" production from quiescent bone surfaces (i.e., minimodeling)[6,7,34,35],

	PFM	DTM	PFM/DTM ratio
%B.Ar	+10	+5*	2x
Tb.Wi	+19	+7*	2.7x
Node/Free Ratio	+3.5	+0.6*	5.8x
Bone balance	+14	+1	14.0

*Net change=mean value of respective parameter in the rats treated with 0.1 μg/kg/d of alfacalcidol for 12 weeks minus the mean value of baseline controls.

Table 6. Comparison of net changes* of static bone mass, architecture and BMU bone balance parameters in cancellous bone of PFM and DTM.

	Prox. Fem. Metaphysis PFM	Lumbar Vertebral Body LVB	Ratio LVB/PFM
%B.Ar	+21%	+51%	2.4
%Er.Pm	-	-82%	-
%Oc.Pm	-48%	-	-
%L.Pm	-51%	-41%	-1.2
%Ob.Pm	-34%	-	-
BMU bone balance	+14%	+41%	2.9

Lumbar vertebral body data from the same animal as in Table 1.

Table 7. Percent differences relative to respective controls following alfacalcidol treatment in selective histomorphometric parameters of cancellous bone in proximal femoral metaphysis and lumbar vertebral body.

which in the current study contributed only 0.085% more bone mass during the last 14 days of treatment. Even though there were only a few "boutons" formed during the last 14 days of treatment, their formation earlier would have contributed to the increased bone mass. In addition, there was a dramatic accumulation of connectivity in terms of increased nodes to free ends ratio (+111%) for the 3-month treatment period. Previously we reported a strong correlation between "boutons" and connectivity[7] confirming Erben's previous report[34] that "bouton" formation was responsible for the improved connectivity. It is apparent that, a time course study is needed to determine which anabolic mechanism (minimodeling or remodeling-dependent bone gain) plays the dominant role in increasing bone mass, as well as whether "boutons" formation directly increases to positive bone balance and the early relationship between "boutons" and increased connectivity.

The comparison of the net changes induced by alfacalcidol treatment in Table 5 allowed us to define why the red marrow PFM gained 2-fold more cancellous bone than the yellow marrow DTM. The alfacalcidol-treated PFM histomorphometric profile better fit the Riggs and Parfitt[44] definition of an anabolic drug that requires both an increase in bone remodeling and a formation phase greater in magnitude than the resorption phase. Although, alfacalcidol treatment depressed both bone resorptions, the magnitude of the depression of bone resorption and formation was less in the PFM than in the DTM; thus, the PFM possesses a higher remodeling rate and larger increase in the amount of bone formation over resorption than in the DTM to allow it to show increased bone mass and improved architecture.

Of interest was our current finding that not all red marrow sites respond to the same degree. A comparison of the red marrow sites at proximal femoral metaphysis (PFM) and the lumbar vertebral body (LVB) found the PFM to be less efficacious than the LVB in adding cancellous bone mass. There was a 2.4 times less increase in cancellous bone mass and 2.9-fold less increased positive BMU balance (Table 7).

Investigators have been focused on fewer osteoblasts and osteoblast lineage precursor cells, poorer vascularity and reduced bone turnover as events contributing to the diminishing of osteoblastogenesis and lack of bone gain. Reduced population of mesenchymal stem cells (MSC) in fatty marrow sites could be one explanation of the cause for the attenuated bone formation response[5,45]. Marrow mesenchymal stem cells, precursors to mesenchymal osteoprogenitor cells (MOPC), are less common at fatty than red marrow sites due in part to the former commitment to the adipocytic rather than the osteoblastic phenotype[46]. It follows that there will be fewer osteoblast lineage cells available for bone formation in yellow marrow skeletal sites. Yellow marrow bone sites are less vascularized than red marrow sites[47-49] contributing fewer circulating osteoblast lineage cells[50-52]. Further, reduced vascularity would suggest a relatively poorer nutrient supply and exposure to lower local concentration of alfacalcidol to osteoblast lineage cells than to osteoblast lineage cells adjacent to more highly vascularized red marrow. In support, mineral apposition rate, an index of osteoblast function, has been reported to be decreased significantly in yellow marrow compared to red marrow sites[2,53]. Additionally, cancellous bone turnover in yellow marrow bone sites are known to be lower than red marrow sites[2,53]. Reduced bone turnover is accompanied by diminished angiogenesis[54], reduction in osteogenic potential cells from pericytes[52] and the outer lining of the bone remodeling compartments[54,55] as well as lower local concentration of bone resorption-derived cytokines and growth factors[56-59], the latter believed to be beneficial in activating osteoblasts to form bone. In summary, these numerous events in the fatty marrow created a less favorable osteoblastogenesis environment linked to reduced osteoblast lineage cells, vascularity and bone turnover that attenuated the cancellous bone formation in yellow marrow DTM to form less bone than in the red marrow PFM.

Common to the red and yellow marrow bone sites are

bone lining cells (BLC) playing a role in bone formation[60,61]. Increasingly reports have shown that BLC are converted into osteoblasts in response to parathyroid hormone[40,41,62-66]. The relative lack of a significant stimulatory effect of alfacalcidol on bone formation at the fatty marrow DTM suggests that BLC may not be the target cells for alfacalcidol, which instead increases proliferation of MSC and MOPC. A similar conclusion was reached by Pun et al.[5] who showed stimulation of bone formation by basic fibroblast growth factor was attenuated and lacking in fatty marrow sites and concluded that the reduced population of MSC and MOPC at fatty marrow sites is probably the most likely explanation for the attenuation of the bone anabolic response. Nevertheless, the activation of bone bouton formation on quiescent bone surfaces in the alfacalcidol-treated red marrow PFM suggests that BLC may yet be a target.

In summary, the results of the current study indicate that the positive balance response that increased lamellar bone mass by alfacalcidol treatment was greatly diminished at a fatty marrow site, such as the distal tibial metaphysis in intact female rats. Whereas, alfacalcidol treatment increased cancellous bone mass at sites with hematopoietic marrow such as the proximal femoral metaphysis (PFM) and previously in proximal tibial metaphysis (PTM) and lumbar vertebral body (LVB)[6,7]. Our findings indicate that alfacalcidol as a potential osteoporosis therapy may be less efficacious in non-vertebral skeletal sites lacking in hematopoietic marrow. In addition, of the two hematopoietic bone marrow sites, the LVB responded more efficaciously than the PFM.

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