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Original Article

Hylonome

Alfacalcidol increases cancellous bone in low turnover, fatty marrow sites in aged, orchidectomized rats

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Abstract

The objectives of this study were to determine the responses of cancellous bone in the distal tibial metaphysis (DTM), a low turnover, fatty (yellow) marrow site, to sham-aged, orchidectomy (ORX) and alfacalcidol treatment in sham-aged and ORX rats. Eighteen-month-old male sham and ORX rats were treated with 0.1 and 0.2 μg/kg alfacalcidol 5 days/wk p.o. for 12 weeks, double fluorescent labeled, and the DTM were processed for bone histomorphometry analyses. The current study found the DTM in sham-aged male rats were resistant to age-related and ORX-induced cancellous bone loss and alfacalcidol-induced bone gain, findings that differ from that in the proximal tibial metaphysis (PTM) and lumbar vertebral body (LVB), two high turnover, red marrow bone sites. However, alfacalcidol treatment increased DTM bone mass in ORX rats where bone turnover was elevated by androgen deficiency. These results in concert with the previously positive findings in red marrow bone sites following alfacalcidol treatment suggest that alfacalcidol is more effective in increasing cancellous bone mass in the skeletal sites with higher bone turnover.

Keywords: Alfacalcidol, Orchidectomy, Histomorphometry, Bone Turnover, Bone Balance

Introduction

Previous studies have shown that the cancellous bone in the distal tibial metaphysis (DTM), an adult, low turnover, yellow (fatty) marrow site, behaves differently than high bone turnover, red (hematopoietic) marrow sites such as the proximal tibial metaphysis (PTM) and lumbar vertebral body (LVB), in responding to aging and ovariectomy (OVX) in female rats. The DTM did not have any age-related and OVX-induced bone loss[1-3], a common finding that usually is seen in the PTM and LVB[4,5]. Interestingly, the DTM did develop immobilization-induced bone loss[2] and responded to parathyroid hormones and prostaglandin-E2 anabolic treatments[1,6]. However, the responses of the DTM to aging and androgen deficiency induced by orchidectomy (ORX) in male rats remain unknown.

Alfacalcidol has been shown to exert dual anti-catabolic and anabolic properties. It increased cancellous bone mass in red marrow sites with relatively higher bone turnover such as LVB and PTM[7-13] and prevented age-related cancellous bone loss in the red marrow bone site of aged male rats[11]. In addition, it prevented cancellous bone loss and restored bone mass in OVX rats[7-10]. Furthermore, alfacalcidol had a better effect in increasing bone mass in the red than in the yellow marrow bone sites in female rats[14]. However, it is unknown whether the DTM would respond to alfacalcidol treatment in a similar manner as the red marrow bone we have studied.

Thus, the objective of the current study was to investigate the responses of cancellous bone in the DTM of male rats to sham-aged, ORX and alfacalcidol treatment. To achieve this objective we did the following analyses on the DTM of sham-aged and ORX rats following vehicle or alfacalcidol treatment: (1) the effects of aging in male rats from 18 to 21 months; (2) the effects of orchidectomy; (3) the effects of alfacalcidol treatment in sham-aged male rats; (4) the effects of alfacalcidol treatment in ORX rats; and (5) the comparative effects of alfacalcidol treatment in sham-aged and ORX rats.

Tian, Chen, Setterberg and Jee have no conflict of interest. Li has a corporate appointment with Pfizer, Inc.

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		Sham-Aged			ORX		
	Baseline	**Vehicle**	**0.1**	**0.2**	**Vehicle**	**0.1**	**0.2**
Serum calcium (mg/dl)	12.44±0.47	12.08±0.34 (-3)	12.72±0.55[b] (2; 5)	13.24±0.48[ab] (6; 10)	12.13±0.33 (-2; 0)	12.69±0.59[bc] (2; 5; 5)	14.17±0.43[abc] (14; 17; 17)
Serum inorganic phosphorus (mg/dl)	7.43±0.88	6.56±0.71[a] (-12)	7.94±0.98[b] (7; 21)	8.28±1.03[b] (11; 26)	6.87±0.74 (-8; 5)	7.18±0.92 (-3; 9; 5)	9.27±0.81[abc] (25; 41; 35)

Mean ± SD; (% change from Baseline; or /and % change from Sham-aged vehicle; or/and % change from ORX-vehicle). [a]$p<$ 0.05 vs. Baseline; [b]$p<0.05$ vs. Sham-aged vehicle; [c]$p<0.05$ vs. ORX-vehicle. The baseline and sham data were published previously[11].

Table 1. Serum Biochemistry.

Materials and methods

Animals and materials

The experimental animals were male Sprague-Dawley rats (Harlan, Indianapolis, IN) that were 18-months of age and weighed an average of 540g at the beginning of the study. The animals were housed at 24°C with a 12:12 h light/dark cycle and 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 of vitamin D_3. The experiments were conducted according to Pfizer's Animal Care and Use Committee approved protocols, and the rats were maintained in accordance with the ILAR (Institute of Laboratory Animal Research) Guide for the Care and Use of Laboratory Animals.

Seventy rats were randomly divided into 7 groups with 10 per group. One group of rats was killed at the beginning of the experiment to serve as baseline controls, and the rest of rats were anesthetized with an intraperitoneal injection of ketamine hydrochloride and xylazine at doses of 50 and 10mg/kg body weight, respectively. Bilateral orchidectomies were performed in three of the groups of rats and the remaining three groups of rats were subjected to sham surgeries. The rats received vehicle (cottonseed oil, Sigma-Aldrich, Inc., St. Louis, MO) or alfacalcidol (Calbiochem, La Jolla, CA) 5 days/week for 12 weeks 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 0.1 and 0.2 μg/ml for 0.1 and 0.2 μg/kg/d group, respectively. All rats were subcutaneously injected with Calcein at a dose of 10 mg/kg (Sigma Chemical Co., St. Louis, MO) on days -12 and -2 prior to sacrifice. At the conclusion of 12 weeks of treatment with alfacalcidol, the rats were fasted 16 hours prior to necropsy. On the day of necropsy, whole blood samples were collected by cardiac puncture immediately after euthanasia by CO_2 asphyxiation for biochemical assessments. Blood samples were centrifuged to obtain the sera, which were stored at -20°C until assay. The right tibiae were harvested and prepared for bone histomorphometry analysis.

Serum biochemistry

Serum calcium (Ca) and inorganic phosphorus (Pi) concentrations were measured with the 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 right 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). Sagittal sections of distal tibiae (DTM) were cut at a 230 μm thickness using a Low Speed Diamond Saw (Isomet, Buehler, Lake Bluff, IL, USA), and then hand ground to a thickness of 20 μm for histomorphometric analyses[15]. All bone measurements were performed using a semi-automatic Image Analysis System (OsteoMeasure, OsteoMetrics Inc. Decatur, GA). Cancellous bone measurements were performed in the area between the former epiphyseal-metaphyseal junction to 3 mm proximal to the junction.

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). 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 (F#: CTF+NTF+2xFTF); 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) and bone formation rate per unit of bone area, tissue area and bone surface (BFR/B.Ar, BFR/T.Ar, BFR/BS)[16-19], and the estimated index of tissue level bone balance (%L.Pm/%Er.Pm)[2,20]. The index of tissue level

		Sham-Aged			ORX		
	Baseline	**Vehicle**	**0.1**	**0.2**	**Vehicle**	**0.1**	**0.2**
Trabecular bone area (%B.Ar, %)	22.49±2.72	22.01±5.84 (-2)	23.30±3.16 (4; 6)	25.73±7.22 (14; 17)	21.40±2.88 (-5; -3)	27.52±5.01$^{abc\#}$ (22; 25; 29; 18)	32.31±8.85^{abc} (44; 47; 51; 26)
Trabecular width (Tb.Wi, (m)	112.21±16.30	118.93±23.62 (6)	106.32±13.62 (-5; -11)	108.66±16.85 (-3; -9)	107.21±14.50 (-4; -10)	120.56±18.10 (7; 1; 12; 13)	135.19±32.45$^{c\#}$ (20; 14; 26; 24)
Trabecular number (Tb.N, #)	2.03±0.25	1.85±0.40 (-9)	2.19±0.13^{b} (8; 18)	2.35±0.43^{b} (16; 27)	2.01±0.25 (-1; 8)	2.29±0.29^{abc} (13; 23; 14; 4)	2.38±0.13^{abc} (17; 28; 18; 1)
Trabecular separation (Tb.Sp, μm)	388.71±55.79	447.12±143.55 (15)	351.05±28.47 (-10; -21)	329.27±83.71^{b} (-15; -26)	396.78±54.63 (2; -11)	327.49±58.69^{abc} (-16; -27; -18; -8)	301.09±43.16^{abc} (-23; -33; -24; -13)
Node to node/total area (NTN/T.Ar, #/mm^2)	1.88±0.59	1.03±0.45^{a} (-45)	1.68±0.60^{b} (-10; 63)	2.13±0.79^{b} (14; 107)	1.45±0.74 (-23; 41)	1.86±0.94^{b} (-1; 80; 28; 10)	2.71±0.43^{abc} (44; 163; 87; 27)
Ratio of nodes number/ Free ends number (N/F)	1.06±0.61	0.80±0.37 (-24)	0.88±0.30 (-17, 10)	1.01±0.49 (-4, 26)	0.71±0.35 (-33, -12)	1.58±0.72$^{bc\#}$ (50, 97; 124; 80)	1.68±0.71$^{bc\#}$ (59, 110; 138; 67)

Mean ± SD; (% change from Baseline; and/or % change from Sham-aged vehicle; and/or % change from ORX-vehicle; and/or % change from same dose of Sham + Alfacalcidol)
$^{a}p<0.05$ vs. Baseline; $^{b}p<0.05$ vs. Sham-aged vehicle; $^{c}p<0.05$ vs. ORX-vehicle; $^{\#}p<0.05$ ORX+Alfacalcidol vs. same dose of Sham+Alfacalcidol.

Table 2. Selective Static Trabecular Bone Histomorphometric Parameters of Distal Tibial Metaphysis.

bone balance was estimated by the ratio of labeled and eroded perimeters (%L.Pm/%Er.Pm). Lastly, the atypical bone formation previously described as bone "boutons"[12-14,21] or "bone buds"[11] were observed and quantitated. Tables 1-4 list the calculated differences in responses in alfacalcidol-treated DTM of sham-aged and ORX rats comparing baseline, sham-aged, and ORX controls. Additionally, the same dose of alfacalcidol-treated ORX and sham-aged rats were compared.

Statistical analysis

Data were presented as mean ± SD for each group. 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 $p<0.05$ on a two-tailed test. In addition, all groups were analyzed with two-way factorial ANOVA (SPSS, Chicago, IL, USA), where the two factors were alfacalcidol and orchidectomy.

	B.B Frequency (#/An)	**B.B.N (#)**
Baseline	1/10	1.00
Sham-vehicle	4/10	1.00±0.00 (0)
Sham - 0.1	7/10	1.43±0.53 (43; 43)
Sham - 0.2	10/10	3.20±2.15 (220; 220)
ORX-vehicle	1/10	1.00 (0; 0)
ORX - 0.1	6/10	1.67±0.82 (67; 67; 67)
ORX - 0.2	7/7	3.71±2.93 (271; 271; 271)

Mean ± SD; (% change from Baseline; and/or % change from Sham-aged vehicle; and/or % change from ORX-vehicle;.
$^{a}p<0.05$ vs. Baseline; $^{b}p<0.05$ vs. Sham-aged vehicle; $^{c}p<0.05$ vs. ORX-vehicle.

Table 3. Occurrence of Bouton Surface in Alfacalcidol- treated Sham-aged and ORX Rats.

Results

Body weight and serum calcium and phosphorous

The mean body weights of all groups remained constant throughout the study (data not shown).

The serum calcium levels did not differ between the baseline and sham-aged vehicle controls, but the 0.1 μg alfacalcidol/kg-treated sham-aged rats significantly increased by 5% versus sham-aged vehicle control and the 0.2 μg/kg-treated sham-aged rats significantly increased by 6% and 10% over the baseline and sham-aged vehicle controls. The serum calcium levels in ORX-vehicle controls did not differ from baseline and sham-aged vehicle controls; however, the 0.1 μg alfacalcidol/kg dose significantly increased calcium levels by 5% compared to both sham-aged vehicle and ORX-vehicle controls while the 0.2 μg/kg dose increased calcium levels by 14, 17 and 17% in terminal ORX'd treated rats compared to baseline, sham-aged

		Sham-Aged			ORX		
	Baseline	**Vehicle**	**0.1**	**0.2**	**Vehicle**	**0.1**	**0.2**
Labeled perimeter (L.Pm, %)	5.38±2.41	8.93±3.98 (66)	3.31±1.38[ab] (-38; -63)	5.03±2.01[b] (-6; -44)	15.87±6.92[ab] (195; 78)	3.97±2.12[bc] (-26; -56; -75; 20)	8.50±4.58[c#] (58; -5; -46; 69)
Mineral apposition rate (MAR, μm/day)	0.56±0.11	0.55±0.07 (-1)	0.53±0.13 (-5; -3)	0.53±0.12 (-4; -4)	0.62±0.05[b] (13; 13)	0.56±0.10 (1; 1; -11; 3)	0.62±0.14 (13; 13; 0; 17)
Bone formation rate (BFR/BS, $\mu m^3/\mu m^2$/dx100)	2.99±1.43	5.09±2.64[a] (70)	1.71±0.69[ab] (-43; -66)	2.79±1.58[b] (-7; -45)	9.84±4.44[ab] (229; 93)	2.24±1.33[bc] (-25; -56; -77; 31)	5.51±3.63 (84; 8; -44; 97)
Bone formation rate (BFR/B.Ar, %/year)	16.99±8.89	27.05±13.95 (59)	10.02±4.78[b] (-41; -63)	16.08±9.60[b] (-5; -41)	55.91±22.59[ab] (229; 107)	11.37±6.48[bc] (-33; -58; -80; 13)	23.22±10.68[c] (37; -14; -58; 44)
Percent Eroded perimeter (%Er.Pm, %)	1.51±0.78	2.61±0.73[a] (73)	0.79±0.29[ab] (-48; -70)	0.93±0.37[b] (-38; -64)	3.81±1.15[ab] (152; 46)	0.85±0.48[abc] (-44; -67; -78; 7)	1.03±0.46[bc] (-32; -61; -73; 10)
Ratio of labeled perimeter/ eroded perimeter (%L.Pm/%Er.Pm)	4.30±2.82	3.60±1.86 (-16)	5.04±3.42 (17; 40)	6.55±4.96 (53; 82)	4.23±1.90 (-2; 17)	6.47±4.31 (51; 80; 53; 28)	11.55±9.53[abc] (169; 220; 173; 76)

Mean ± SD; (% change from Baseline; and/or % change from Sham-aged vehicle; and/or % change from ORX-vehicle; and/or % change from same dose of Sham + Alfacalcidol)
[a]$p<0.05$ vs. Baseline; [b]$p<0.05$ vs. Sham-aged vehicle; [c]$p<0.05$ vs. ORX-vehicle; [#]$p<0.05$ ORX + Alfacalcidol vs. same dose of Sham + Alfacalcidol.

Table 4. Selective Trabecular Bone Histomorphometric Parameters of Distal Tibial Metaphysis.

and ORX-vehicle controls (Table 1).

Serum phosphorus levels significantly dropped 12% in sham-aged vehicle rats while the 0.1 and 0.2 μg alfacalcidol-treated sham-aged animals had significant increases of 21% and 26% versus sham-aged vehicle controls. In contrast, significant increased serum phosphorous levels were limited to the 0.2 μg/kg alfacalcidol-treated ORX rats to 25% and 41% compared to beginning and sham-aged vehicle rats and a significant elevation of 35% from ORX-vehicle rats (Table 1).

Effects of aging in sham-aged vehicle rats

Cancellous bone area remained constant while there were significant decreases in node to node population (NTN/T.Ar, -45%) and a non-significant reduction in ratio of node to free end (N/F, -24%; $p=0.277$) compared to baseline control (Table 2). Four out of 10 sham-aged DTM had one bone bouton site per slide (Table 3).

Histomorphometrically, there was a near significant increase in bone formation-based bone turnover (BFR/B.Ar, 59%, $p=0.071$) with significant increases in index of bone resorption (%Er.Pm, 73%) and surfaced-based bone formation rate (BFR/BS, 70%) indicating a tendency for an index of negative bone balance (-16%) compared to baseline controls (Table 4).

Effects of androgen deficiency induced by orchidectomy

Cancellous bone mass, micro-architecture and bone bouton production did not differ from baseline and sham-aged vehicle controls (Tables 2 and 3), even though there were significant increases in bone turnover (BFR/B.Ar, 107%), bone resorption (%Er.Pm, 46%) and bone formation parameters (%L.Pm, 78%; MAR, 13%; BFR/BS, 93%), leading to non-significant increase in index of positive tissue level (17%) compared to sham-aged vehicle controls (Table 4). One out of 10 ORX DTM had one bone bouton site per slide (Table 3).

Effects of 0.1 μg alfacalcidol/kg in sham-aged rats

Cancellous bone mass and micro-architecture were unchanged from baseline control but select micro-architectural parameters like trabecular number (Tb.Wi, 18%) and node to node population (NTN/T.Ar, 63%) were increased significantly compared to sham-aged vehicle control rats (Table 2). Bone bouton production was higher in alfacalcidol-treated rats (7 out of 10 versus one and 4 out of 10 from baseline and sham-aged vehicle controls) (Table 3).

Histomorphometrically, there were significant decreases in bone turnover (BFR/B.Ar, -63%), bone resorption (%Er.Pm, -70%) with a similar magnitude of indices of bone formation (%L.Pm, -63% and BFR/BS, -66%) contributing to a non-significant estimate of an increase in index of positive tissue level (%L.Pm/%Er.Pm, 40%) versus sham-aged vehicle controls (Table 4).

Effects of 0.2 μg alfacalcidol/kg in sham-aged rats

Cancellous bone mass and micro-architecture were non-significantly increased from baseline and sham-aged vehicle controls; however, trabecular number (Tb.N, 27%), trabecular separation (Tb.Sp, -26%) and node to node population

Variables	Two-way ANOVA Results (*p* value)		
	Alfacalcidol	ORX	Interaction
%B.Ar (%)	0.013	NS (0.212)	NS (0.098)
Tb.Wi (μm)	NS (0.949)	NS (0.829)	0.031
Tb.N (#)	0.002	NS (0.176)	NS (0.738)
Tb.Sp (μm)	0.003	NS (0.154)	NS (0.684)
NTN/T.Ar (#/mm^2)	0.025	NS (0.195)	NS (0.590)
N/F	0.003	NS (0.530)	0.012
%O.Pm (%)	<0.001	NS (0.052)	NS (0.177)
%L.Pm (%)	<0.001	0.008	0.027
%Er.Pm (%)	<0.001	0.012	0.022
MAR (μm/d)	NS (0.169)	NS (0.141)	NS (0.363)
BFR/B.Ar (%/yr)	<0.001	0.002	0.004
BFR/BS (μm^3/μm^2/dx100)	<0.001	0.005	0.021
%L.Pm/%Er.Pm	NS (0.068)	NS (0.300)	NS (0.682)

Table 5. Two-way ANOVA Analysis 0.1 μg Alfacalcidol/kg-treated Distal Tibial Metaphysis of Orchidectomized Rats.

(NTN/T.Ar, 107%) differed significantly from sham-aged vehicle controls (Table 2). Bone bouton production occurred in all animals at 3.2±2.2 sites per slide (Table 3).

Histomorphometrically, there were significant decreases in bone turnover (BFR/B.Ar, -41%), bone resorption (%Er.Pm, -64%), and bone formation parameters (%L.Pm, -44%; BFR/BS, -45%); but marked, non-significant, increased indication of positive tissue bone balance (%L.Pm/%Er.Pm, 82%, $p=0.277$) versus sham-aged vehicle controls (Table 4).

Effects of 0.1 μg alfacalcidol/kg in ORX rats

There were significant increases in cancellous bone mass (%B.Ar, 22%) and select micro-architectural parameters (trabecular number, 13%; trabecular separation, -16%) compared to baseline controls. Additionally, there were significantly more trabecular bone mass (%B.Ar, 25%), number (Tb.N, 23%), node to node population (NTN/T.Ar, 80%), ratio of node to free end (N/F, 97%), and less separation (Tb.Sp, -27%) versus sham-aged vehicle controls (Table 2). Six out of ten ORX DTM had 1.7±0.8 bone boutons per slide (Table 3).

Histomorphometrically, there was significantly decreased bone turnover (BFR/B.Ar, -58%) in combination with significantly decreased bone resorption (%Er.Pm, -67%), more than decreased bone formation parameters (%L.Pm, -56% and BFR/BS, -56%), and a trend toward an increase in the index of positive tissue bone balance (%L.Pm/%Er.Pm, 80%, $p=0.070$) compared to sham-aged controls (Table 4).

Factorial ANOVA showed the interaction of ORX and 0.1 μg alfacalcidol/kg had significant influence on the changes in trabecular width, ratio of nodes to free ends, bone turnover, bone formation and eroded perimeter (Table 5), but no interaction on bone bouton production (data not shown).

Effects of 0.2 μg alfacalcidol/kg in ORX rats

Compared to baseline controls, this treatment significantly increased cancellous bone mass (%B.Ar, 44%) and improved select micro-architectures in trabecular number (Tb.Wi, 17%), trabecular separation (Tb.Sp, -23%) and node to node population (NTN/T.Ar, 44%) while it tended to increase ratio of node to free end (N/F, 59%, $p=0.071$) (Table 2). All 7 out of 7 treated ORX DTM showed 3.7±2.9 bone boutons per slide (Table 3).

Compared to terminal sham-aged vehicle and ORX-vehicle control rats, significant histomorphometrical findings were restricted to depressed bone resorption (%Er.Pm, -61% and -73%) and increased index of positive tissue bone balance (%L.Pm/%Er.Pm, 220% and 173%). In addition, bone turnover was significantly lower (BFR/B.Ar, -58%) versus ORX controls (Table 4).

Factorial ANOVA showed the interaction of ORX and 0.2 μg alfacalcidol/kg had significant influence on changes in trabecular width, ratio of nodes to free ends, bone resorption and bone turnover (Table 6), but no interaction on bone bouton production (data not shown).

Differences in responses to 0.1 μg alfacalcidol/kg between ORX and sham-aged rats

Significant differences were limited to increased cancellous bone mass by 18% and ratio of node to free end by 80% (Table 2). No difference was observed in bone bouton production (Table 3). There were non-significant differences in increases of select histomorphometric indices in bone turnover (BFR/B.Ar, 13%), bone resorption (%Er.Pm, 7%), bone formation (BFR/BS, 31%) and index of positive tissue bone balance (%L.Pm/%Er.Pm, 28%) compared to sham-aged rats treated with the same 0.1 μg alfacalcidol dose (Table 4).

Variables	Two-way ANOVA Results (p value)		
	Alfacalcidol	ORX	Interaction
%B.Ar (%)	0.001	NS (0.166)	NS (0.097)
Tb.Wi (μm)	NS (0.232)	NS (0.316)	0.031
Tb.N (#)	<0.001	NS (0.419)	NS (0.561)
Tb.Sp (μm)	0.001	NS (0.143)	NS (0.907)
NTN/T.Ar (#/mm^2)	<0.001	0.025	NS (0.717)
N/F	0.001	NS (0.078)	0.021
%O.Pm (%)	NS (0.328)	0.013	NS (0.822)
%L.Pm (%)	0.001	0.002	NS (0.278)
%Er.Pm (%)	<0.001	0.015	0.035
MAR (μm/d)	NS (0.735)	0.018	NS (0.761)
BFR/B.Ar (%/yr)	<0.001	0.001	0.042
BFR/BS (μm^3/μm^2/dx100)	0.004	0.001	NS (0.349)
%L.Pm/%Er.Pm	0.004	NS (0.102)	NS (0.119)

Table 6. Two-way ANOVA Analysis 0.2 μg Alfacalcidol/kg-treated Distal Tibial Metaphysis of Orchidectomized Rats.

Differences in responses to 0.2 μg alfacalcidol/kg treatment between ORX and sham-aged rats

There was a non-significant increase in cancellous bone mass (26%, p=0.112), while significant differences were limited to increased trabecular thickness (Tb.Wi, 24%) and ratio of node to free end (N/F, 67%) (Table 2). No differences were detected in bone bouton production (Table 3). Significant increases in histomorphometric parameters were restricted to percent labeled perimeter (69%), although BFR/BS was nearly significant (97%, p=0.051). Again, increases of other select indices of histomorphometric parameters were non-significantly different from the sham-aged; but at a higher level in the 0.2 μg dose treated ORX rats where: bone turnover (BFR/B.Ar, 44%), bone resorption (%Er.Pm, 10%) and index of positive tissue bone balance (76%) increased compared to sham-aged rats treated with the same 0.2 μg dose (Table 4).

Discussion

This study was designed to investigate the responses of the cancellous bone in the distal tibial metaphysis (DTM), a low turnover, fatty (yellow) marrow site, to aging, orchidectomy and alfacalcidol treatment in aged male rats. The data yielded the following new findings: (1) age-related changes in the DTM were restricted to the loss in trabecular connectivity (reduced node to node population) in contrast to losses in both cancellous bone mass and bone architecture in the proximal tibial metaphysis and the lumbar vertebral body (high turnover, red marrow sites)[22]; **(2)** orchidectomy did not induce cancellous bone loss or architecture in the DTM but exhibited significantly increased bone turnover with accompanying bone balance in equilibrium (the magnitude of bone formation and resorption is equal to the magnitude of bone formation); (3) alfacalcidol-treatment dose-dependently increased serum calcium and phosphorus in sham-aged and ORX rats; (4) alfacalcidol treatment in sham-aged rats dose-dependently prevented age-related reduction in trabecular connectivity (reduced node to node population), decreased cancellous bone turnover, increased bone bouton production, but did not cause any significant bone gain; (5) alfacalcidol treatment in ORX rats significantly increased cancellous bone mass as well as improved micro-architecture. This effect was accompanied with increased bone bouton production, dose-responsively reduced bone turnover, decreased bone resorption more than bone formation and at the 0.2 μg/kg dose maintained bone formation resulting in bone gain; and (6) alfacalcidol induced bone boutons on cancellous bone of DTM in both sham and ORX rats, a phenomenon that was previously reported only in high turnover, red marrow sites in adult female and aged male rats[11-14].

Contrary to the aged-related loss of cancellous bone mass and poorer micro-architecture seen in the cancellous bone of the proximal tibial metaphysis and lumbar vertebral body in aged male rats (21 months vs. 18 months of age)[11], the age-related change in DTM from the same age animals was limited to a reduction in node to node population. However, it had in common to the other red marrow sites[11], that the DTM showed an age-related increase in bone turnover but differed with no change in bone balance status with bone resorption and formation in equilibrium. Our findings indicate that, unlike in the skeletal sites with relatively high turnover red marrow where testosterone depletion induces cancellous bone osteopenia[22], testosterone deficiency induced by ORX does not cause bone loss in the DTM. Orchidectomy did markedly increase bone turnover in the DTM, which suggests increased bone turnover alone does not induce cancellous bone loss but it is the imbalance from more bone resorption than formation leading to a negative tissue bone balance in red marrow skeletal sites. In contrast, in the DTM, bone balance was in equilibrium and bone mass

remained unchanged; the same condition also occurred in ovariectomized rats[1,3].

In both alfacalcidol-treated sham-aged and ORX rats, the bone mass and micro-architectural changes occurred as a result from a combination of depressed bone resorption and formation, in which the depression in bone resorption was greater than the depression in bone formation or decreased resorption without any effect on bone formation, resulting in a positive bone balance and gain. These findings are consistent with previous findings in relatively young rats[7-10,12-14,23-27]. In alfacalcidol treated DTM of sham rats, the preventive effect in age-related impairment in cancellous bone connectivity was likely due to the decrease in bone turnover and increase in bone bouton production. In contrast, in the alfacalcidol treated ORX rats, the magnitude of the suppressed bone resorption exceeded that of bone formation, resulting in significant increases in positive bone balance and gain. Apparently, the histomorphometric profile of alfacalcidol-treated DTM in ORX rats was in the mode of a classical anabolic bone drug defined by Riggs and Parfitt - a drug that requires both an increase in bone remodeling and formation phase greater in magnitude than the resorption phase[28]. The 0.2 μg/kg alfacalcidol-treated ORX rats differed from the sham rats receiving the same treatment with higher bone turnover, maintaining bone formation and bone resorption leading to marked increased positive bone balance and bone gain (Tables 2 and 4). Bone formation parameters in the 0.2 μg/kg alfacalcidol-treated ORX rats trended toward significant increases (%L.Pm, +58%, p=0.064; BFR/BS, +84%, p=0.064) compared to baseline and not different from sham-aged vehicle controls, indicating that the treatment did not increase, but at least maintained the overall bone formation in the sham-aged vehicle control level. The combination of maintained bone formation and decreased bone resorption produced a significant increase in positive bone balance between bone formation and resorption resulting in a net bone gain and improved architecture. In addition, the alfacalcidol-treated DTM in ORX rats induced similar increases in bone gain seen in alfacalcidol-treated red marrow LVB sites in aged male rats[11].

It is unclear how orchidectomy enhanced the positive effect of alfacalcidol in the DTM. One explanation is the response to the stimulation of bone turnover by ORX, which creates a more favorable environment (more BMU creations) for stimulating osteoblastogenesis. The significant interaction between alfacalcidol treatment and ORX in bone turnover and bone resorption as demonstrated by the factorial 2-way ANOVA analysis supports this hypothesis. Orchidectomy-stimulated bone turnover may be accompanied by increased angiogenesis associated with increased osteogenic cells from pericytes[29] outer lining of bone remodeling compartments[30,31] and circulating osteoblast lineage cells[29,32,33] as well as a relatively better nutrient supply; exposure to higher local concentration of alfacalcidol and higher local concentration of bone resorption-derived cytokines and growth factors[34-37] that are believed to be beneficial in activating osteoblasts to form bone from osteoblast lineage cells and bone lining cells[38,39]. Consistently, significant interactions were found between alfacalcidol and orchidectomy in increasing bone formation bone area-based (BFR/B.Ar, bone formation-based bone turnover) as assessed by the factorial 2-way ANOVA analysis.

Another finding in the study is the first report of the occurrence of alfacalcidol-induced atypical pattern of bone formation, bone boutons, in cancellous bone of the yellow marrow DTM in sham and ORX rats. Erben et al. first reported bone bouton production in red marrow, bone sites in adult rats receiving pharmacological doses of calcitriol[21,40] Subsequently, it was found in the alfacalcidol-treated cancellous bone in PTM and LV of aged male and adult female rats[11-13], but not in relatively younger rats[7]. In addition, Liu et al. previously reported a strong positive correlation between the amount of bone boutons and increased connectivity[13] that may influence the maintenance and increase in connectivity with alfacalcidol treatment in the current study.

Based on bone boutons' micro-anatomical features, they are a result of the accumulation of focal packets of bone formation on bone surfaces stimulated by alfacalcidol treatment mainly by minimodeling, where bone formation occurs on smooth cement lines (i.e., quiescent bone surfaces) without prior osteoclastic bone resorption[41,42]. Mimimodeling occurs in tibial and vertebral cancellous bone in aging rats as well as in transiliac biopsy specimens from patient who underwent total hip arthroplasty and parathyroid hormone treatment[43,44]. This focal formed bone at the minimodeling sites in human specimens are similar to some of the bone boutons observed in the rats treated with alfacalcidol. It is not known how the bone boutons evolved. Unknown changes in the microenvironment, duration of treatment, etc. may play a role in the initiation of bone boutons[44]. Since there were similar incidences of bone bouton formation in both sham and ORX rats, it indicated that ORX, did not play a role in initiation of bone bouton formation.

In the current study, we could not separate the direct and indirect effects of alfacalcidol because of the increase in serum calcium induced by alfacalcidol stimulation of calcium absorption. High calcium stimulates bone formation and inhibits resorption *in vitro*[45]. It is well established to decrease bone turnover *in vivo*[46,47]. Thus, the decrease in bone resorption and maintenance of bone formation may be partially due to the high serum calcium.

In summary, the current study showed that the cancellous bone in the DTM, a low turnover, yellow marrow bone site responded to aging, orchidectomy and alfacalcidol treatment differently than that in PTM and LVB, two high turnover, red marrow bone sites. In another words, cancellous bone in the DTM of aged male rats was insensitive to age-related and androgen-induced bone loss or alfacalcidol treatment. However, it responded to alfacalcidol treatment positively when its bone turnover was elevated by androgen deficiency. These results in concert with the previously positive findings in red marrow bone sites following alfacalcidol treatment suggest that alfacalcidol is more effective in increasing cancellous bone mass in the skeletal sites with higher bone turnover.

References

1. Ma YF, Ke HZ, Jee WSS. Prostaglandin E2 adds bone to a cancellous bone site with a closed growth plate and low bone turnover in ovariectomized rats. Bone 1994;15:137-46.
2. Ijiri K, Ma YF, Jee WSS, Akamine T, Liang X. Adaptation of non-growing former epiphysis and metaphyseal trabecular bones to aging and immobilization in rat. Bone 1995;17(Suppl.4):S207-12.
3. Li M, Shen Y, Qi H, Wronski TJ. Comparative study of skeletal response to estrogen depletion at red and yellow marrow sites in rats. Anat Rec 1996;245:472-80.
4. Wronski TJ, Dann LM, Horner SL. Time course of vertebral osteopenia in ovariectomized rats. Bone 1989;10:295-301.
5. Wronski TJ, Dann LM, Scott KS, Cintrón M. Long-term effects of ovariectomy and aging on the rat skeleton. Calcif Tissue Int 1989;45:360-6.
6. Li M, Liang H, Shen Y, Wronski TJ. Parathyroid hormone stimulates cancellous bone formation at skeletal sites regardless of marrow composition in ovariectomized rats. Bone 1999;24:95-100.
7. Shiraishi A, Takeda A, Masaki T, Higuchi Y, Uchiyama Y, Kubodera N, Sato K, Ikeda K, Nakamura T, Matsumoto T, Ogata E. Alfacalcidol inhibits bone resorption and stimulates formation in an ovariectomized rat model of osteoporosis: distinct actions from estrogen. J Bone Miner Res 2000;15:770-9.
8. Shiraishi A, Higashi S, Masaki T, Saito M, Ito M, Ikeda S, Nakamura T. A comparison of alfacalcidol and menatetrenone for the treatment of bone loss in an ovariectomized rat model of osteoporosis. Calcif Tissue Int 2002;71:69-79.
9. Nishikawa T, Ogawa S, Kogita K, Manabe N, Katsumata T, Nakamura K, Kawaguchi H. Additive effects of combined treatment with etidronate and alfacalcidol on bone mass and mechanical properties in ovariectomized rats. Bone 2000;27:647-54.
10. Li M, Healy DR, Simmons HA, Ke HZ, Thompson DD. Alfacalcidol restores cancellous bone in ovariectomized rats. J Musculoskelet Neuronal Interact 2003;3:39-46.
11. Li M, Healy DR, Li Y, Simmons HA, Su M, Jee WSS, Shen VW, Thompson DD. Alfacalcidol prevents age-related bone loss and causes an atypical pattern of bone formation in aged male rats. J Musculoskelet Neuronal Interact 2004;4:22-32.
12. Chen HY, Tian XY, Liu XQ, Setterberg RB, Li M, Jee WSS. Alfacalcidol stimulated focal bone formation on the cancellous surface and increased bone formation on the periosteal bone surface of the lumbar vertebra of adult female rats. Calcif Tissue Int 2008;82:127-36.
13. Liu XQ, Chen HY, Tian XY, Setterberg RB, Li M, Jee WSS. Alfacalcidol treatment increases bone mass and strength from anti-catabolic and anabolic effects on cancellous and cortical bone in intact female rats. J Bone Miner Metab 2008;26:425 35.
14. Tian XY, Liu XQ, Chen HY, Setterberg RB, Li M, Jee WSS. Greater efficacy of alfacalcidol in the red than in the yellow marrow skeletal sites in adult female rats. J Musculoskeletal Neuronal Interact 2008;8:257-66.
15. Jee WSS, Inoue J, Jee KW, Haba T. Histomorphometric assay of the growing long bone. In: Takahashi H, editor. Handbook of Bone Morphology. Niigata Japan: Nishimura; 1983. p. 101-103.
16. Parfitt AM, Mathews CHE, Villanueva AR, Kleerekoper M, Frame B, Rao DS. Relationships between surface, volume and thickness of iliac trabecular bone in aging and in osteoporosis. Implications for the microanatomic and cellular mechanisms of bone loss. J Clin Invest 1983;72:1296-409.
17. Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PF, Ott SM, Recker RR. Bone histomorphometry: Standardization of nomenclature, symbols and units. J Bone Miner Res 1987;2:595-610.
18. Compston JE, Mellish RWE, Garrahan NJ. Age-related changes in iliac crest trabecular microanatomic bone structure in man. Bone 1987;8:289-92.
19. Compston JE, Mellish RWE, Croucher P, Newcombe R, Garrahan JH. Structural mechanisms of trabecular bone loss in man. Bone Miner 1989;6:339-50.
20. Ma Y, Chen YY, Jee WSS, Ke HZ, Ijiri K. Co-treatment of PGE_2 and risedronate is better than PGE2 alone in the long-term treatment of ovariectomized-induced osteopenic rats. Bone 1995;17(Suppl.4):S267-72.
21. Erben RG. Vitamin D analogs and bone. J Musculoskelet Neuronal Interact 2001;2:59-69.
22. Erben RG, Eberle J, Stahr K, Goldberg M. Androgen deficiency induces high turnover osteopenia in aged male rats: a sequential histomorphometric study. J Bone Miner Res 2000;15:1085-98.
23. Erben RG, Weiser H, Sinowatz F, Rambeck WA, Zucker H. Vitamin D metabolites prevent vertebral osteopenia in ovariectomized rats. Calcif Tissue Int 1992;50:228-36.
24. Erben RG, Bromm S, Stangassinger M. Therapeutic efficacy of 1α,25-dihydroxyvitamin D_3 and calcium in osteopenic ovariectomized rats: evidence for a direct anabolic effect of 1α,25-dihydroxyvitamin D3 on bone. Endocrinology 1998;139:4319-28.
25. Erben RG, Bante U, Birner H, Stangassinger M. Prophylactic effects of 1,24,25-dihydroxyvitamin D_3 on ovariectomy-induced cancellous bone loss in the rat. Calcif Tissue Int 1997;60:434-40.
26. Erben RG, Mosekilde L, Thomsen JS, Weber K, Stahr K, Leyshon A, Smith SY, Phipps R. Prevention of bone loss in ovariectomized rats by combined treatment with risedronate and 1α,25-dihydroxyvitamin D_3. J Bone Miner Res 2002;17:1498-511.
27. Tanaka Y, Nakamura T, Nishida S, Suzuki K, Takeda S, Sato K, Nishii Y. Effects of a synthetic vitamin D ana-

log, ED-71, on bone dynamics and strength in cancellous and cortical bone in prednisolone-treated rats. J Bone Miner Res 1996;11:325-36.
28. Riggs BL, Parffit AM. Perspective. Drugs used to treat osteoporosis: the critical need for a uniform nomenclature based on their action on bone remodeling. J Bone Miner Res 2005;20:177-84.
29. Brandi ML, Collin-Osdoby P. Vascular biology and the skeleton. J Bone Miner Res 2006;21:183-92.
30. Eriksen EF, Eghbali-Fatourechi GZ, Khosla S. Remodeling and vascular spaces in bone. J Bone Miner Res 2007;22:1-6.
31. Hauge EM, Qvesel D, Eriksen EF, Mosekilde L, Melsen F. Cancellous bone remodeling occurs in specialized compartments lined by cells expressing osteoblastic markers. J Bone Miner Res 2001;16:1575-82.
32. Kuznetsov SA, Mankani MH, Gronthos S, Satomura K, Bianco P, Robey PG. Circulating skeletal stem cells. J Cell Biol 2001;153:1133-40.
33. Eghbali-Fatourechi GZ, Lamsam J, Fraser D, Nagel D, Riggs BL, Khosia S. Circulating osteoblast-lineage cells in humans. N Engl J Med 2005;352:1959-66.
34. Centrella M, McCarthy TL, Canalis E. Transforming growth factor-beta and remodeling of bone. J Bone Joint Surg Am 1991;73:1418-28.
35. Baylink DJ, Finkelman RD, Mohan S. Growth factors to stimulate bone formation. J Bone Miner Res 1993;8(Suppl.2):S565-72.
36. Mundy GR, Bonewald LF. Role of TGF beta in bone remodeling. Ann NY Acad Sci 1990;593:91-7.
37. Karsdal MA, Martin TJ, Bollerslev J, Christiansen C, Henriksen K. Are nonresorbing osteoclasts sources of bone anabolic activity? J Bone Miner Res 2007;22:487-94.
38. Miller SC, Bowman BM, Smith JM, Jee WSS. Characterization of endosteal bone-lining cells from fatty bone sites in adult beagles. Anat Rec 1980; 198:163-73.
39. Miller SC, Jee WSS. The bone forming cells: Origin, distribution proliferation and differentiation. In: Takahashi HE, editor. Bone Morphometry. Niigata: Nishimura and London: Smith-Gordon; 1988. 29, 36.
40. Erben RG, Kohn B, Weiser H, Sinowatz F, Rambeck WA. Role of vitamin D metabolites in the prevention of the osteopenia induced by ovariectomy in the axial and appendicular skeleton of the rat. A Ernährungswiss 1990;29:229-48.
41. Jee WSS, Tian XY, Setterberg RB. Cancellous bone minimodeling-based formation: a Frost, Takahashi legacy. J Musculoskelet Neuronal Interact 2007;7:232-9.
42. Erben RG. Trabecular and endocortical bone surfaces in the rat: modeling or remodeling? Anat Rec 1996;246:39-46.
43. Kobayashi S, Takahashi HE, Ito A, Saito N, Nawata M, Horiuchi H, Ohta H, Ito A, Iorio R, Yamamoto N, Takaoka K. Trabecular minimodeling in human iliac bone. Bone 2003;32:163-9.
44. O'Donnell S, Moher D, Thomas K, Hanley DA, Cranney A. Systematic review of the benefits and harms of calcitriol and alfacalcidol for fractures and falls. J Bone Miner Metab 2008;26:531-42.
45. Brown EM. Calcium-sensing receptor. In: Favus MJ, editor. Primer on Metabolic Diseases and Disorders on Mineral Metabolism, Fifth Edition. Washington DC, ASBMR; 2003. p. 111-117.
46. McKane WR, Khosla S, Egan KS, Robins SP, Burritt MF, Riggs BL. Role of calcium intake in modulating age-related increases in parathyroid function and bone resorption. J Clin Endocrinol Metab 1996;81:1699-703.
47. Blair HC, Simonet S, Lacey DL, Zidi M. Osteoclast biology. In: Marcus R, Feldman D, Nelson D, Rosen CJ, editors. Osteoporosis, Third Edition, Volume I. Amsterdam, Elsevier Academic Press; 2008. p. 151-168.