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Action of Calciotropic Hormones on Bone Metabolism – Role of Vitamin D_3 in Bone Remodeling Events

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Abstract: Vitamin D_3 is known to have immunosuppressive effects that can be beneficial for treatment of immune disorders and transplant rejection, however therapeutic application is limited due to hypercalcemia and hypercalcuria. The goal of our studies was to explore both the acute and steady state effects of vitamin D_3 on bone remodeling as potential limiting factors to broader use of vitamin D_3 in the clinic. Vitamin D_3 was evaluated for its skeletal effects in both thyroparathyroidectomized (TPTx) and intact rat models. In TPTx rats, deprivation of thyroid and parathyroid hormones and calcitonin creates a low state of bone modeling and remodeling ideal for evaluation of changes imposed by drug intervention. The use of both models allowed for discrimination of individual (TPTx) versus combined (intact) effects of calciotropic hormones on bone and calcium metabolism. Our studies have confirmed the limitations of using vitamin D_3 for treatment/co- treatment of immune disease in humans due to the intrinsic hypercalcemic properties of the hormone, and also highlighted the potential of vitamin D_3 to negatively impact skeletal integrity due to excessive bone remodeling driven by bone resorption. Taken together our data emphasize the importance of including biomarkers of bone remodeling as an integral part of clinical and preclinical studies using vitamin D_3 to treat immune disorders and suggest the need for co-treatment with an antiresorptive agent to counteract hypercalcemia and deterioration of bone.

Key words: Calcitriol, Parathyroid hormone, Calcitonin, Rats, Thyroparathyroidectomy, Biomarkers

INTRODUCTION

The anti-proliferative, pro-differentiating and immunosuppressive effects of hormone 1,25-dihydroxyvitamin D_3 (Vit D_3, calcitriol) are well defined[1]; however, in order to achieve maximal immunosuppressive activities, concentrations of vitamin D_3 are required that may be associated with hypercalcemia. A significant body of evidence suggests that vitamin D_3 elicits a suppressive effect on the immune system through a complex interaction involving antigen-presenting cells and T-cells[2,3]. In addition, VDR agonists are shown to improve transplantation tolerance[4] and prolong allograft survival in several organs[5,6]. While hypercalcuria and hypercalcemia are widely recognized as a limiting factor when using vitamin D_3 to treat autoimmune diseases, attention has focused on recruitment of dietary calcium by vitamin D_3 and not the skeletal effects. Vitamin D_3 has effects on skeletal bone remodeling and at high doses can increase bone resorption and release of calcium thereby contributing to systemic hypercalcemia and hypercalcuria.

In addition to its mechanical function, the skeleton serves as a reservoir for calcium and phosphorus, minerals that are crucial for proper functioning of many cells in the body. Critical calcium homeostasis is maintained by powerful endocrine control mechanisms consisting of calciotropic hormones. The calciotropic hormones: vitamin D_3, parathyroid hormone (PTH) and calcitonin (CT) regulate calcium absorption from the intestine, calcium excretion and re-absorption from kidneys and calcium deposition or release from bone. It is estimated that 99% of the total body calcium resides within the crystalline structure in bone while the remaining 1% is rapidly exchanged between bone and extra-cellular fluids. The skeleton includes both cortical bone that primarily provides mechanical support and protection of vital organs and cancellous bone that has both a metabolic and a mechanical role. In rats, cancellous bone at the tibial metaphysis has a predominantly metabolic role and is characterized by a high turnover rate, whereas cancellous bone at the tibial epiphysis has a predominantly mechanical role and is characterized by a low turnover rate[7,8]. The process of calcium deposition in bone is carried out by osteoblasts, cells that derive from stromal osteoprogenitor cells and synthesize new bone matrix. Osteoblasts are characterized by high alkaline phosphatase activity, receptors for PTH and 1,25-dihydroxyvitamin D_3, and the ability to synthesize a number of noncollagenous bone matrix proteins[9,10]. Bone resorption is the process of calcium release from bone matrix and is carried out by osteoclasts, multinucleated cells derived from hematopoietic precursors in the bone marrow and other hematopoietic organs. Osteoclasts are characterized by

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receptors for calcitonin and the ability to carry out solubilization of bone mineral and hydrolysis of dense bone collagen[11,12].

All three calciotropic hormones have very distinct functions and work in concert to maintain calcium homeostasis. PTH is the peptide hormone secreted rapidly by parathyroid chief cells in response to changes in blood calcium and is involved in both bone resorption and bone formation. The effect of PTH on bone resorption is dominant and continuous administration of PTH leads to a net release of calcium from bone. Osteoclasts lack PTH receptors, thus the action of PTH on osteoclasts is mediated through binding of PTH to osteoblasts and production of osteoblastic surface proteins, macrophage colony-stimulating factor (M-CSF) and RANK ligand (RANKL)[13,14]. Non-follicular cells of the thyroid gland called C-cells that originate from the neuronal crest secrete calcitonin. The secretion rate of CT is tightly regulated by the calcium ion concentration within C-cells. When administered acutely, CT decreases tubular reabsorption of calcium and impairs bone resorption by acting directly on osteoclasts through its receptor[15]. The major role of Vitamin D_3 in calcium homeostasis is stimulation of intestinal absorption of calcium and phosphate, thereby providing the proper microenvironment for bone mineralization. Vitamin D_3 is a major transcriptional regulator of the two most abundant bone matrix proteins; it represses the synthesis of type I collagen and induces the synthesis of osteocalcin[16-19]. Vitamin D_3 also promotes the differentiation of osteoclasts from monocyte-macrophage stem cell precursors *in vitro* and at high doses increases osteoclastic bone resorption *in vivo* by stimulating osteoblast production of osteoclast-differentiating factor (RANKL)[20-21].

The goal of our studies was to differentiate the effect of Vitamin D_3 on intestinal calcium adsorption versus bone remodeling and to explore both the acute and steady state effect of vitamin D_3 on calcium and bone metabolism in the situation where dietary calcium is readily available. In order to better understand its pharmacology, we evaluated skeletal effects of vitamin D_3 at the tibial metaphysis and epiphysis since these two sites contain cancellous bone with distinct metabolic and mechanical properties. Emphasis was put on utilizing various biomarkers of bone and calcium metabolism to determine skeletal involvement when vitamin D_3 is used to treat autoimmune disease.

MATERIAL AND METHODS

Materials: Bovine 1-34 PTH fragment (Sigma, St. Louis, MO) was dosed at approximately 0.0017 mg/kg/hr as follows; the PTH was prepared in sterile saline at a concentration of 30 μg/ml and dosed at 8 μl/hour by continuous infusion using a mini-pump, Alzet model 2001D (Durect Corporation, Cupertino, CA). Mini-pumps were prepared according to manufacturers' directions using aseptic technique and were pre-incubated prior to placement in the animal to ensure that PTH was actively released starting at time 0. Animals were anesthetized using isofluorane for aseptic surgical placement of the mini-pumps in the subcutaneous compartment at the nape of the neck. Salmon Thyrocalcitonin (Sigma, St. Louis, MO) was prepared in sterile water at a concentration of 50 μg/ml and dosed at 80 μg/kg by subcutaneous injection at time 20 hours. Internal and external data[22] was used to guide dose selection of Calcitriol, 1,25-Dihydroxyvitamin D_3 (Calbiochem, Germany) that was prepared in corn oil (Sigma, St. Louis, MO) and dosed according to the individual study descriptions.

Animal models: Experiments were carried out in male Sprague-Dawley (CD) rats, either intact, TPTx or age-matched controls, that were 12 weeks old at the time of the experiments. All animal procedures were approved by the Institutional Animal Care and Use Committee and conformed to NIH standards established in the "Guidelines for the Care and use of Laboratory Animals". Removal of the thyroid and parathyroid glands was done surgically at Charles River labs (Wilmington, MA) 10 days prior to the start of the experiment. During the surgical recovery period, the TPTx rats were kept on regular rat chow and 1% calcium gluconate water. Upon arrival at Pfizer, TPTx rats were acclimatized for one day before start of the experiment, while normal rats were acclimatized for one week before start of the experiment. All rats were housed in a room maintained at 23 ± 1°C, 55 ± 5% humidity with a 12-hour light/dark cycle. Body weight was measured in all rats upon arrival at Pfizer, before starting the experiment, after 24 hours, and before euthanasia at day 7 of the study period.

Experimental Diets: The following experimental diets purchased from Harlan Teklad (Madison, WI) were used: A) Calcium deficient diet (TD.95027) containing 0.007% Ca and 0.4% Phosphorus; B) Vitamin D and calcium deficient diet (TD.05176) containing 0.01% Ca and 0.6% Phosphorus and C) Vitamin D deficient but calcium enriched diet (TD.04124) containing 1% Ca, 0.6% Phosphorus. Vitamin D deficient diets had < 1IU of vitamin D per kg diet. Diets were supplied in pelletized form and fed *ad libitum* and/or prepared for oral gavage (2 ml/animal) by mixing 100 g of diet with 80 mls of water and grinding with mortar and pestle to a paste. In the case of the calcium enriched diet gavage, this preparation resulted in a calcium dose of ~80 mg/kg.

Serum and urine chemistry and assays: Blood, 0.5 ml was collected at various time points by retro-orbital bleed for serum chemistry (calcium and phosphorus) measurements and for determination of bone turnover biomarkers. Animals were lightly anesthetized during the bleed procedure with CO_2/O_2. Chemistry endpoints were analyzed using a Hitachi 917

auto analyzer (Roche, Indianapolis, IN). Commercially available ELISA kits were utilized for determination of osteocalcin (OC; Nordic Bioscience, Denmark), specific epitope of collagen type 1 alpha 1 (CTX; Nordic Bioscience, Denmark), osteoclast-derived tartrate-resistant acid phosphatase form 5b (TRAP; Suomen Bioanalytikka, Finland), parathyroid hormone (PTH; Immutopics Inc., San Clemente, CA) and free sRANKL (Free sRANKL; American Laboratory Co., Windham, NH). Urine samples were analyzed for creatinine, phosphorus and calcium using a Hitachi 917 auto-analyzer. Urine samples were also analyzed for deoxypyridinolone as a marker of bone resorption using an ELISA kit (Quidel, San Diego, CA).

Bone histology and structure: Bone histology was performed at the proximal tibia using a method described earlier[23]. In brief, at the end of the study, tibias were excised and placed in 4°C, 10% neutral buffered formalin (Decal, Congers NY) for three days to allow for fixation. Tibias were then washed with cold running tap water for one hour then placed in 5% EDTA at 4°C for decalcification, and paraffin processed. Sections were cut at 5μm and stained with hematoxylin and eosin (H&E). Osteoclasts were stained with Tartrate-Resistant Acid Phosphatase (TRAP) using a Leukocyte Acid Phosphatase Kit #387-A (Sigma Diagnostics, Inc., St. Louis, MO). Bone remodeling was evaluated at proximal tibial metaphysis (high turnover) and at proximal tibial epiphysis (low turnover). *In vitro* μ-CT analysis was performed using a μCT-40® computed tomography system (Scanco Medical, Bassersdorf, Switzerland). The methods employed have been described previously[24]. The method allows reproducible positioning of the bone specimen for scanning. Samples were scanned at high-resolution mode with a source energy kVp of 55 and mA of 145. Micro-CT measurements are done to evaluate cancellous bone structure in studies when dosing was carried out for 7 days.

Treatment protocols: Study 1 was conducted using TPTx rats randomized to one of the following groups based on body weight and/or surgical status: 1. Intact control = normal, age matched CD rats, no treatment; 2. TPTx controls = TPTx rats, no treatment; 3. TPTx + PTH = TPTx rats treated with constant, 24 hour infusion of human PTH (0.0017 mg/kg/hr) by mini-pump, and 4. TPTx + PTH + CT = TPTx rats receiving PTH infusion and injected subcutaneously with salmon Calcitonin (80 μg/kg) at time 20. Time course was as follows: Day 1, rats arrive at facility, body weight recorded, transfer to Ca deficient diet (TD.95027) and RO water. Day 2, Time –2 hours, baseline bleeds collected; Time 0, PTH mini-pump were implanted and rats were transferred to metabolism cages for urine collection; Time 20, bleed followed by CT injection to group 4; Time 24, bleed, urine harvest and termination for all groups.

Studies 2 & 3 were conducted using intact (study 2) or TPTx (study 3) CD rats that were randomized to one of four groups based on body weight. All treatment groups were given Ca and D_3 deficient diet for washout (TD.05176), select treatment groups were maintained on the Ca and D_3 deficient diet for the duration of the experiment while calcium dosed groups received Ca enriched/D_3 deficient diet (TD.04124) as indicated during the experimental period. In order to ensure consistency and minimize individual variations in amount and timing of dietary calcium during the 24-hour experiments, animals were given food bolus (BID) by oral gavage. Treatment groups were as follows: Vehicle Controls = treated with corn oil vehicle, 1 ml/kg, orally 2x/day; Calcium = treated orally with Ca enriched food bolus 2x/day; Vit. D_3 = treated orally 2x/day with 5.0 μg/kg of vitamin D_3 and Calcium + Vit. D_3 = treated 2x/day with Ca food bolus and with 5.0 μg/kg of vitamin D_3. Time course was as follows: Day -2, body weight recorded, transfer to Ca and D_3 deficient diet (TD.05176); Day 1, Time –1 hours, baseline bleeds; Time 0, vitamin D_3 or vehicle dose and transfer all animals to metabolism cages to start urine collection; Time 1, food bolus with Ca enriched/D_3 deficient diet (TD.04124); Time 3, bleed; Time 6, bleed, end urine collection, 2nd vitamin D or vehicle dose; Time 7 hours, 2nd food bolus and give experimental diet ad libitum throughout the experiment; Time 24 hours, bleed and termination.

Studies 4 & 5 were conducted using intact (study 4) or TPTx (study 5) CD rats that were randomized to one of four groups based on body weight. All treatment groups were given Ca and D_3 deficient diet for washout (TD.05176) and Ca enriched/D_3 deficient diet (TD.04124) as indicated during the experimental period. Treatment groups were as follows: Vehicle Controls = treated orally with corn oil vehicle, 1 ml/kg/day; High dose D_3 = treated orally with 5.0 μg/kg/day of vitamin D_3; Medium dose D_3 = treated orally with 1.0 μg/kg/day of vitamin D_3; and Low dose D_3 = treated orally with 0.2 μg/kg/day of vitamin D_3. Time course was as follows: Day -1, body weight recorded, transfer to Ca and D_3 deficient diet (TD.05176); Day 1, Time –1 hours, baseline bleeds; Time 0, vitamin D_3 or vehicle dose and transfer all animals to metabolism cages to start urine collection; Time 1, food bolus with Ca enriched/D_3 deficient diet (TD.04124); Time 6, bleed, end urine collection and give experimental diet (TD.04124) ad libitum throughout the experiment; Time 24, bleed; Day 2-6, dose; Day 6, six hour urine set; Day 7, Time 0, bleed then dose; Time 3 post dose, termination and necropsy.

Statistical analysis: Data from biochemical analyses are presented as mean ± S.D. Statistical analysis of the data was performed by unpaired Student's t-test. Differences were considered to be statistically significant with a p-value of < 0.05.

RESULTS

Results from **study 1** revealed that TPTx surgery resulted in hypocalcemia with significantly lower serum calcium levels in TPTx controls compared to intact rats (Figure 1, Time –2 hours). By the end of the study period, continuous infusion of PTH increased serum calcium to normocalcemic values (Figure 1; Group 3). Subcutaneous injection of calcitonin rapidly dropped serum calcium in PTH treated rats (Group 4).

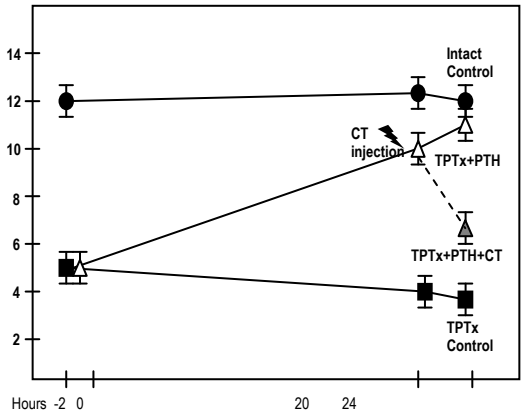
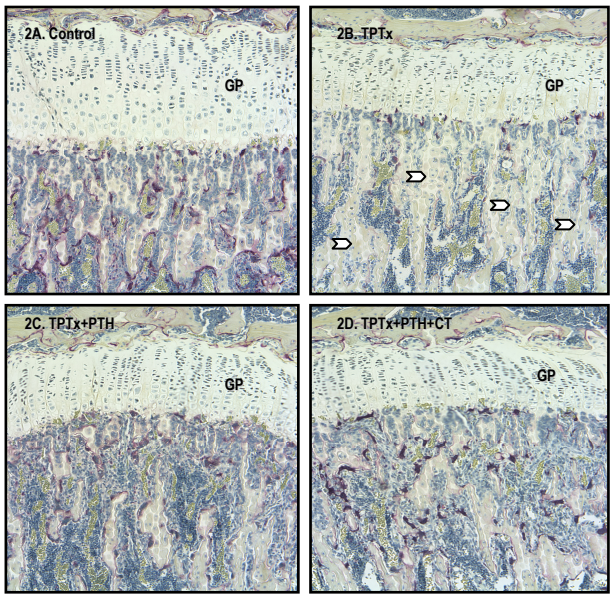


Figure 1. The effect of PTH and CT on serum calcium levels in TPTx rats over 24 hours. Data are mean ± SD, 6-8 rats per experimental group.

TRAP histology data revealed reduced growth-plate thickness and inactive bone surfaces in TPTx control rats relative to intact controls. Continuous administration of PTH caused activation of osteoclasts, while calcitonin injection induced detachment of osteoclasts from bone surfaces in PTH treated rats (Figures 2 and 3).

Results from **study 2** in intact rats showed significantly lower serum levels of PTH in vitamin D_3 treated groups relative to control (Figure 4). Dietary calcium supplementation had no effect on serum PTH levels. Both groups of vitamin D_3 treated rats had higher serum osteocalcin levels; the group receiving vitamin D_3 alone had higher osteocalcin levels than the group receiving vitamin D_3 and calcium. Osteoclast activity was higher only in the group of rats receiving vitamin D_3 alone. Calcium alone had no effect on biomarkers of bone remodeling. Simultaneous dosing of vitamin D_3 and calcium resulted in hypercalcemia at 24 hours. Rats treated with vitamin D_3 alone showed a mild increase in serum calcium, while dosing with calcium fortified diet alone had no effect on serum calcium.

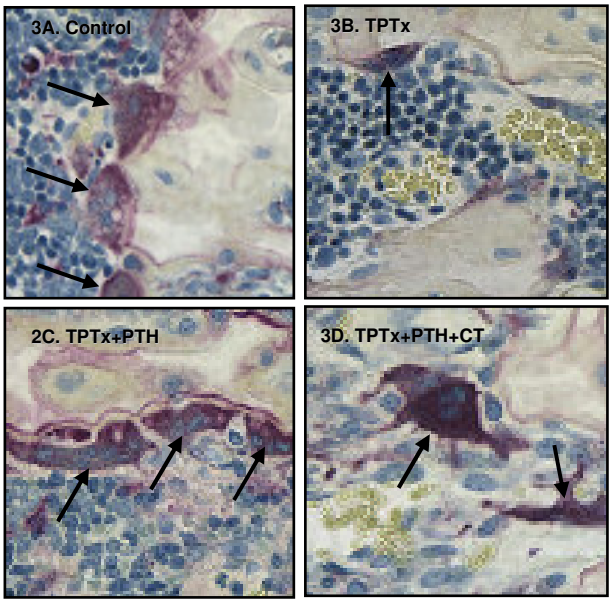
Results from **study 3** using TPTx rats showed increased levels of TRAP and osteocalcin in the groups treated with Vitamin D_3 regardless of dietary calcium (Figure 5). Administration of vitamin D_3 and calcium proved very effective in correcting hypocalcemia, while dosing with vitamin D_3 alone elicited only a moderate increase in serum calcium. TPTx rats seems to be more sensitive to vitamin D_3 and calcium treatment compared to intact animals with an average increase in serum calcium of 56% in TPTx compared to a 12% average increase in intact rats. Similar to results obtained in intact rats, administration of calcium lactate alone did not change any of the measured parameters of calcium metabolism.



Figures 2A- 2D depicts TRAP stained osteoclasts (red) located in primary and secondary spongiosa of proximal tibial metaphysis. TPTx control rats (2B) exhibit thinner growth plates and fewer osteoclasts in primary and secondary spongiosa relative to intact controls (**2A**). Despite impaired growth in TPTx rats the lack of bone resorption resulted in thicker trabeculae (arrowheads). Continuous administration of PTH increased osteoclast number and size resulting in marked resorption of tibial spongiosa (**2C**), but with no effect on growth plate width. Single injection of calcitonin affected activity of osteoclasts, but did not affect their number (2D). TRAP histochemistry; magnification x10.

Results from **study 4** using intact rats revealed hypercalcemia by day seven, regardless of vitamin D_3 dose deployed. In addition, the 5-µg/kg dose induced hypercalcemia following a single injection as demonstrated by the significant increase in serum calcium at 24 hours (Figure 6). Hypercalcemia was accompanied by a corresponding increase in the serum calcium and phosphorus ratio (data not shown) and similar changes in urine chemistry indicative of hypercalcuria (data not shown). Osteoclast activity as indicated by changes in serum TRAP levels, was decreased in the high dose group at 24 hours, but after 7 days of dosing, bone resorption was significantly higher in both the high and medium dose groups, while the low dose group was not significantly different from

vehicle controls (Figure 7). Serum levels of s free RANKL in vitamin D_3 treated rats showed a dose responsive decrease, in both intact and TPTx rats (Table 1). Serum osteocalcin levels were significantly higher than controls in the mid and high dose vitamin D_3 groups after 7 days of dosing and also at 24 hours after a single dose in the high vitamin D_3 group (Figure 8). Osteocalcin levels in the low dose group remained similar to control values through the entire experiment.



Figures 3A-3D depicts differences in osteoclast morphology between controls and TPTx rats treated with PTH and/or CT. TPTX control rats (3B) exhibited fewer and smaller osteoclasts relative to intact controls (3A). Continuous administration of PTH increased osteoclast number, size and activity as judged by the cell size, number of nuclei and depth of the resorption pits (3C). Single injection of CT did not affect osteoclast number but caused detachment of osteoclasts from bone surfaces resulting in osteoclast deactivation (3D). TRAP histochemistry; mag. x20.

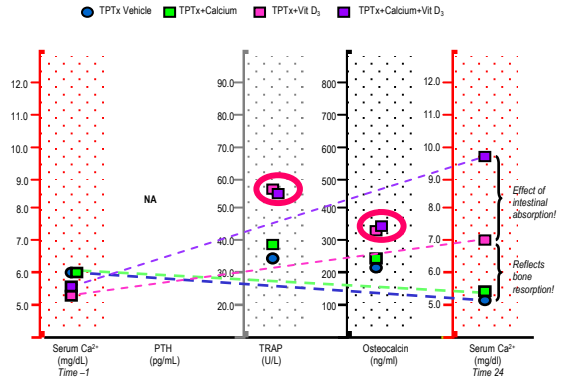


Figure 4 depicts serum levels of calcium at –1 and calcium, TRAP (osteoclast activity) and osteocalcin (osteoblast activity) at 24 hours post administration of vitamin D_3 ± calcium food bolus in intact CD rats. Data are mean ± SD, 6-8 rats per experimental group.

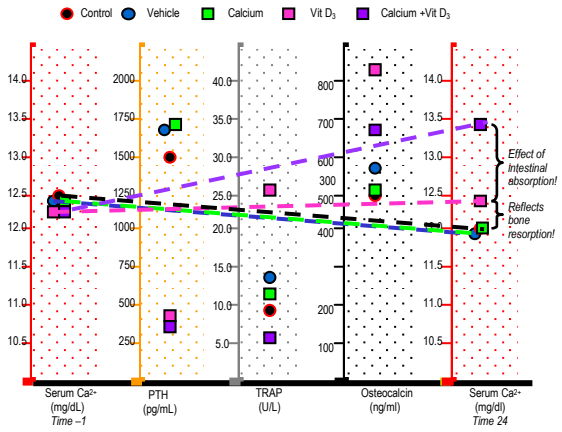


Figure 5 depicts serum levels of calcium at –1 and calcium, TRAP (osteoclast activity) and osteocalcin (osteoblast activity) at 24 hours post administration of vitamin D_3 ± calcium food bolus in TPTx rats. Data are mean ± SD, 6-8 rats per experimental group.

	RANKL (pmol/L)	RANKL (pmol/L)
Treatment group	**Intact Rats**	**TPTx Rats**
Vehicle	1.04±0.48	0.467±0.18
Vit. D_3 (5 µg/kg)	0.49±0.14	0.000±0.00
Vit. D_3 (1 µg/kg)	0.63±0.05	0.033±0.49
Vit. D_3 (0.2 µg/kg)	1.27±01.10	0.162±0.14

Table 1: Serum levels of free RANKL in intact and TPTx rats following 7 days of dosing with vitamin D_3**.**

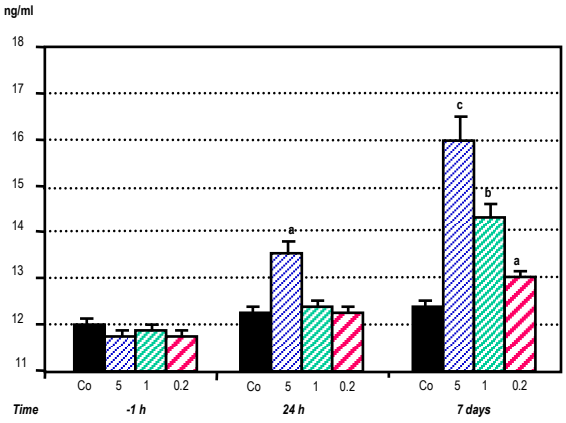


Figure 6 depicts serum calcium levels at –1 and 24 hours, and 7 days following administration of high (5µg/kg/day), medium (1µg/kg/day) and low (0.2 µg/kg/day) dose of vitamin D_3 in intact rats. Data are mean ± SD, 6-8 rats per experimental group. Statistical significance using Students'-test is indicated as: $^{a}p<0.05$; $^{b}p<0.01$ and $^{c}p<0.001$ relative to intact controls.

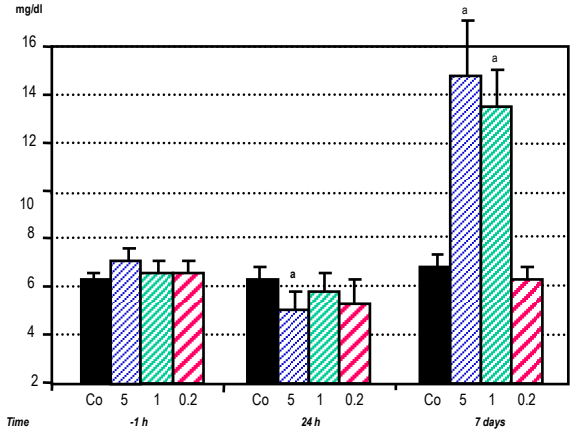


Figure 7 depicts change in serum TRAP levels at –1 and 24 hours, and 7 days following administration of high (5 µg/kg/day), medium (1 µg/kg/day) and low (0.2 µg/kg/day) dose of vitamin D_3 in intact rats. Data are mean ± SD, 6-8 rats per experimental group. Statistical significance using Students't-test is indicated as: [a]p<0.001 relative to intact controls.

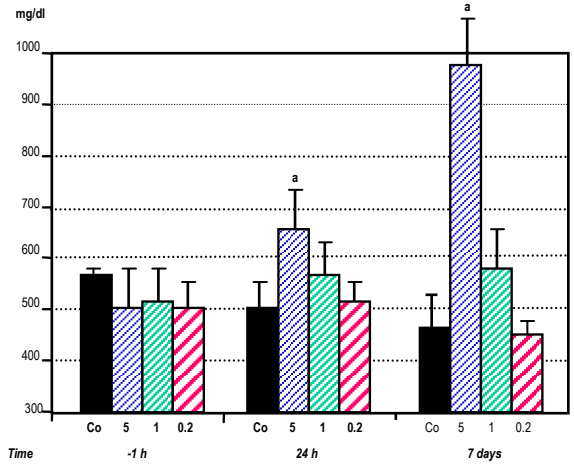


Figure 8 depicts change in serum osteocalcin levels at –1 and 24 hours, and 7 days following administration of high (5 µg/kg/day), medium (1 µg/kg/day) and low (0.2 µg/kg/day) dose of vitamin D_3 in intact rats. Data are mean ± SD, 6-8 rats per experimental group. Statistical significance using Students't-test is indicated as: [a]p<0.05 and [b]p<0.01 relative to intact controls.

TRAP histology data revealed a significant increase in bone resorption at the tibial epiphysis, but not at the tibial metaphysis in vitamin D_3 treated rats compared to controls (Figure 9). As a result, tibial metaphyses of vitamin D_3 treated rats showed more trabeculae relative to controls (Figure 10). Finally, vigorous bone formation can be seen throughout the epiphyses of vitamin D_3 treated rats compared to vehicle controls (Figure 10). Although differences in cancellous bone parameters measured by micro-CT did not reach statistical significance, there is an obvious trend in vitamin D_3 treated rats towards increased bone volume, connectivity, SMI index and trabecular number relative to control rats. At the same time, trabecular thickness in vitamin D_3 treated rats was decreased compared to control rats (Table 2; Figure 11).

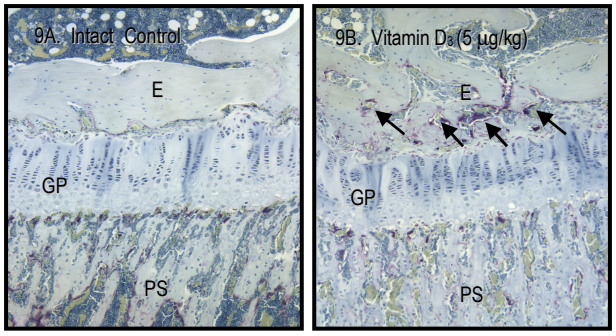


Figure 9 depicts TRAP stained osteoclasts (dark red) in demineralized, paraffin embedded proximal tibia. Intact controls (9A) exhibited numerous osteoclasts in the primary spongiosa (PS) underneath the growth plate (GP), but very few osteoclasts at the epiphysis (E). Intact rats treated with 5 µg/kg of vitamin D_3 (9B) showed thinner growth plate and more bone in the primary spongiosa due to fewer osteoclasts relative to intact controls. In contrast to "quiescent" epiphysis in control rats, vitamin D_3 treated rats exhibit vigorous resorption of trabecular bone at epiphysis (arrows). TRAP histochemistry; magnification x10.

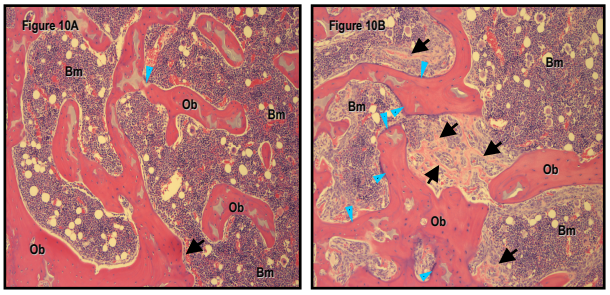


Figure 10 depicts H&E stained cancellous bone at proximal tibial epiphysis from control rat (10A) and rat treated with 5 µg/kg of vitamin D_3 (10B). While control rats exhibit low bone remodeling with most of bone surfaces being covered with bone lining cells, intensive bone remodeling can be seen throughout the epiphysis of vitamin D_3 treated rats with numerous newly formed trabeculae covered by osteoblasts (arrows) and with numerous sites of old bone matrix covered with osteoclasts (arrowheads). Magnification x10; Ob – old bone; Bm – bone marrow.

Results from **study 5** using TPTx rats are similar to those described for intact rats (data not shown). Summary results from all studies presented in table 3 depicts changes in serum biomarkers of calcium and bone metabolism between 24 hours and 7 days following administration of vitamin D_3 in both intact and TPTx rats.

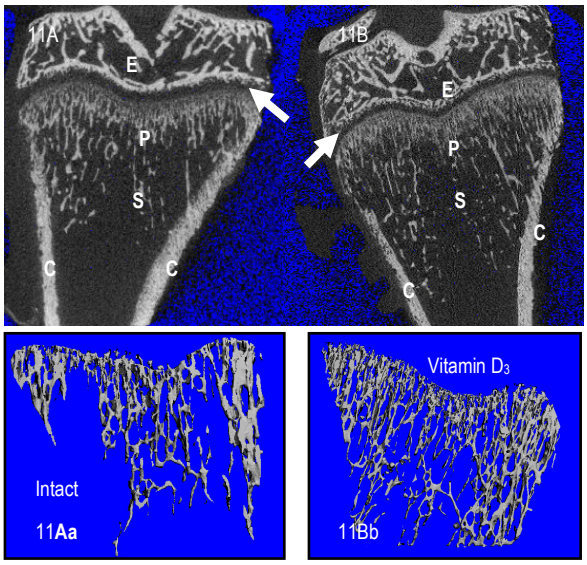


Figure 9 depicts 2D (11A and 11B) images of proximal tibia and 3D (11Aa and 11Bb) images of cancellous bone from proximal tibial metaphyses taken *ex vivo* by micro-CT to demonstrate effect of high dose vitamin D_3 treatment on cancellous and cortical bone and growth plate cartilage. Please note more, but thinner trabeculae in both primary (PS) and secondary (SS) spongiosa of vitamin D_3 treated rats relative to controls (9Aa and 11Bb). Also, trabecular network at epiphysis (E) appears to be thinner and more porous in vitamin D_3 relative to control rats. Similarly, cortical bone (CB) in treated rat looks thinner and more porous relative to control. Arrows indicate growth plate that is visibly thicker in control compared to treated rat indicating possible toxic effect of vitamin D_3 on growth plate chondrocytes.

Parameter	**Intact** Vehicle	***Vit. D₃*** *(5 μg/kg)*	***Vit. D₃*** *(1 μg/kg)*	***Vit. D₃*** *(0.2 μg/kg)*
BV (mm^3)	0.369±0.09	0.546±0.06	0.513±0.04	0.486±0.04
Conn D (1/ mm^3)	38.58±6.01	144.61±57.88	79.93±39.93	56.15±12.12
SMI (1)	2.38±0.17	2.54±0.23	2.31±0.36	2.25±0.16
Tb.N (1/mm)	4.731±0.09	5.205±0.12	4.845±0.19	4.721±0.14
Tb.Th (mm)	0.043±0.002	0.037±0.002	0.042±0.002	0.041±0.001
Tb.Sp (mm)	0.213±0.006	0.198±0.004	0.212±0.009	0.212±0.007

Table 2. Describes structural 3-dimensional cancellous bone parameters evaluated *ex vivo* at proximal tibial metaphysis (secondary spongiosa) using micro-CT. Data from intact rats harvested after 7 days of dosing.

DISCUSSION

There is a great deal of similarity between the human and rodent skeletal response to calcium depletion/repletion[25,26]. *In vivo* calcium metabolism and bone remodeling studies can be difficult to interpret because of the network of mineral and hormonal interactions that are involved. Rats that have undergone surgical removal of the thyroid and parathyroid glands are useful for investigational research in studying the pathophysiology of human hypoparathyroidism, hypothyroidism, calcium and phosphate homeostasis and bone metabolism and particularly for evaluation of compounds with the potential to inhibit bone resorption or elicit bone formation[27-36]. We explored the utility of the TPTx rat with carefully controlled dietary vitamin D_3 and calcium as an in vivo model that allows investigation into the action of vitamin D_3 on bone remodeling events in total absence of PTH and CT. We also investigated and compared the action of vitamin D_3 on bone remodeling in a more clinically relevant, intact rat model.

In order to minimize the effects of bone remodeling induced by growth, we used sexually mature, 3-month old rats for all studies. We found an approximate 9% difference in body weight between age-matched, intact CD and TPTx rats at baseline (allowing a 10-day surgical recovery period). We attribute this difference to the known effect of reduced longitudinal growth due to hypothyroidism in rats that have undergone TPTx surgery[37]. TPTx rats, when compared to controls, demonstrated significant hypocalcemia that was corrected by continuous administration of PTH. The PTH induced calcium release was attributed to resorption since intestinal calcium was limited by feeding a calcium deficient diet. A single dose of CT to rats receiving PTH infusion rapidly decreased serum calcium levels and caused detachment of osteoclasts, overriding PTH induced bone resorption. This data provides an elegant example of direct (CT) vs. indirect (PTH) regulation of bone resorption[37,38]. Despite reduced production of primary spongiosa underneath the growth plate in tibias from TPTx rats, they exhibited more bone at this skeletal site compared to intact controls due to lack of PTH mediated bone resorption and reduced remodeling. The lack of PTH and CT, as well as use of diet to control D_3 and calcium sources has proven a successful method for creating quiescent bone surfaces in TPTx rats, ideal for assessing bone remodeling following drug intervention.

After completing in-house validation of the TPTx rat model, we focused our studies on establishing the acute effect of vitamin D_3 on bone remodeling and calcium homeostasis in the presence or absence of PTH and CT. Vitamin D_3 plays a pivotal role in maintaining calcium homeostasis primarily through its action on intestine and kidney, but it also quickly promotes synthesis of osteocalcin by osteoblasts thereby trapping and storing calcium in bone. Regulation of bone resorption by vitamin D_3 is less well defined since bone resorbing cells lack the vitamin D_3 receptor implying

that vitamin D_3 regulates bone resorption indirectly through its action on osteoblasts[39,40].

24 hours				
INTACT	**Ca^{2+}**	**PTH**	**OC**	**TRAP**
Vehicle	↔	↔	↔	↔
5 μg/kg	↑	↓↓	↑	↓
1 μg/kg	↔	↓	↔	↓
0.2 μg/kg	↔	↔	↔	↓
7 days				
Vehicle	↔	↔	↔	↔
5 μg/kg	↑↑↑	↓↓↓↓	↑↑	↑↑↑
1 μg/kg	↑↑	↓↓↓	↑	↑↑
0.2 μg/kg	↑	↓	↔	↔
24 hours				
TPTx	**Ca^{2+}**	**PTH**	**OC**	**TRAP**
Vehicle	↔	**na**	↔	↔
5 μg/kg	↑	**na**	↑	↑↑
1 μg/kg	↑	**na**	↔	↑
0.2 μg/kg	↔	**na**	↔	↔
7 days				
Vehicle	↔	**na**	↔	↔
5 μg/kg	↑↑↑	**na**	↑↑	↑↑↑
1 μg/kg	↑↑	**na**	↑	↑↑
0.2 μg/kg	↑	**na**	↔	↑

Table 3. Summary of changes in serum level of calcium, parathyroid hormone, osteocalcin and TRAP in intact and TPTx rats after 24 hours and seven days of dosing with vitamin D_3.

Our studies in intact rats confirmed down regulation of PTH by vitamin D_3 as described earlier in rats and humans[27,41-44]. In both TPTx and intact rats administered a vitamin D deficient but calcium enriched diet, no change in serum or urinary calcium was observed suggesting that endogenous synthesis of vitamin D_3 in laboratory rats is too low to correct hypocalcemia through intestinal absorption of calcium. In both TPTx and intact rats administered vitamin D_3 alone, an increase in serum calcium was observed that was attributed to osteoclast mediated bone resorption and not intestinal absorption since rats in this group received no calcium in their diet and exhibited elevated TRAP activity in their serum. Our data support earlier findings in humans and TPTx rats where chronic administration of 1,25-dihydroxyvitamin D_3 was used to normalize serum calcium[28,45-46]. Combination treatment with vitamin D_3 and dietary calcium increased serum calcium in both models bringing TPTx rats closer to normocalcemic levels and causing hypercalcemia in intact rats. In acute, 24 hour studies TPTx rats treated with either vitamin D_3 alone or with vitamin D_3 and calcium exhibited elevated TRAP activity, while in studies using intact rats only rats treated with vitamin D_3 alone showed elevated serum TRAP. Intact rats treated with both D_3 and calcium showed no change in serum TRAP, indicating that intestinal absorption of calcium is the primary mechanism of the acute hypercalcemia observed. On the contrary, in both TPTx and intact rats, chronic dosing of vitamin D_3 and calcium elicited similar increases in serum TRAP. Vitamin D_3 also induced osteocalcin synthesis in both models regardless of supplementation with dietary calcium. Administration of vitamin D_3 alone, in intact rats seemed to be the most effective promoter of osteocalcin synthesis, probably as a mechanism of calcium conservation by storage of calcium in bone in the situation where the supply of dietary calcium is minimal. This body of data demonstrates that vitamin D_3 regulates calcium homeostasis not only through intestinal absorption of calcium, but also through regulation of bone remodeling. Furthermore, results from the TPTx studies suggest that the described effects of vitamin D_3 on intestine and bone are independent of PTH and CT.

Based on results from short-term experiments we expanded study duration to 7 days to better distinguish between the acute and steady state effects of vitamin D_3 on bone metabolism and to better model the clinical situation. Similar to effects seen in humans, more chronic dosing of vitamin D_3 caused hypercaluria and hypercalcemia in both rat models used[45,46]. Results from 7-day experiments in intact rats indicate that the hypercalcemia observed in the low dose group is caused entirely by increased intestinal absorption of calcium, since bone resorption in this group remained unchanged. We also observed a dose proportional decrease following vitamin D_3 treatment, in serum RANKL in both intact and TPTx rats that may indicate better utilization of RANKL by osteoclast precursors in vitamin D_3 treated rats. RANKL, the receptor ligand for RANK is expressed by bone-forming osteoblasts, providing the mechanism whereby bone formation and bone resorption are coupled suggesting critical role of osteoblasts in regulation of bone resorption[47-50]. Furthermore, cytokines and hormones that regulate function of osteoblasts also regulate bone resorption and the balance between RANKL and OPG. It has been proposed that alterations of the RANKL/OPG ratio are critical in the pathogenesis of bone diseases caused by increased bone resorption or altered bone formation such as osteoporosis, hyperparathyroidism, osteolytic bone metastases, myeloma, and several others[51]. After evaluating several assays of bone resorption we favored the use of serum TRAP and RANKL over serum CTX or urinary DpD assays, because they better reflected osteoclast differentiation and activity and highly correlated with histological and micro-CT findings. Additionally, down-regulation of collagen type I synthesis by vitamin D_3 may interfere with CTX and DpD assays when assessing bone resorption. Histology findings paralleled serum findings in vitamin D_3 treated rats, clearly pointing to specific locations of bone resorption. Surprisingly, the most intense bone

resorption was seen at the tibial epiphysis known to be composed of trabecular bone with a strong mechanical role and slow turnover rate[7,8,52]. Simultaneously, bone resorption at the tibial metaphysis was less intense even though this skeletal site contains metabolic bone characterized by a high turnover rate. Therefore, we propose that two parallel processes occurred within the same bone as the result of vitamin D_3 overdose; one being formation of new trabeculae in all sites with cancellous bone, and second, resorption of the old trabecular network regardless of its mechanical relevance. Structural analysis of cancellous bone at the tibial metaphysis confirmed that despite increases in trabecular volume, connectivity and trabecular number parameters, thickness of the trabecular network decreased indicating deterioration of the old bone due to vitamin D_3 treatment. There is a general agreement that cancellous bone is more reactive than cortical bone in response to altered metabolic environments, mainly because cancellous bone has a greater surface-to-volume ratio as well as greater contact with bone marrow. Our results support the existence of an earlier suggested feedback mechanism in mammalian skeletons that control bone remodeling as a function of material quantity and mechanical usage[53,54]. Since mechanical demands on the skeleton in our rats remained unchanged, the excessive accumulation of bone induced by vitamin D_3 resulted in resorption driven bone remodeling leading to deterioration of bone structure and strength[54-56].

Our studies have confirmed the limitations of using vitamin D3 for treatment/co-treatment of autoimmune disease in humans due to the intrinsic hypercalcemic properties of the hormone. Vitamin D_3 has been tested in numerous animal models of autoimmune diseases including the nervous system[57], joints[58], bowel[59], kidneys[60] and skin[61], but very little attention has been given to how treatment affected bone. The most plausible explanation for this oversite is that historically, vitamin D_3 has been portrayed as improving bone formation while hypercalcemia was blamed on increased intestinal absorption of calcium. Here, we show that short-term, high dose treatment with vitamin D_3 negatively impacts skeletal integrity due to excessive bone remodeling driven by bone resorption. The described effects of vitamin D_3 occur even in the absence of PTH or CT.

CONCLUSIONS

Our findings emphasize the importance of including biomarkers of bone remodeling as an integral part of clinical and preclinical studies when using vitamin D_3 to treat autoimmune disorders. These biomarkers should be selected with care to adequately address both bone formation and bone resorption events. Combination therapy with antiresorptives should be considered when using high doses of vitamin D_3 to treat autoimmune disease since this co-therapy may help to achieve the therapeutic index of vitamin D_3 and preserve skeletal integrity. Finally, preclinical use of the described animal models that allow strict control of dietary regimens should help to establish optimal treatment modalities and develop clinical strategies to facilitate the use of vitamin D_3 in the clinic.

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