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Discordant effects of vitamin D deficiency in trabecular and cortical bone architecture and strength in growing rodents☆

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ABSTRACT

We have previously shown that vitamin D deficiency in young male rats results in significant reduction in femoral trabecular bone volume (BV/TV). However, the effects of vitamin D deficiency and its impact on other relevant skeletal sites remain unclear. Ten week old male Sprague–Dawley rats were fed various levels of vitamin D_3 (2, 4, 8, and 12 IU/day) with standard Ca (0.4%) until 30 weeks of age and achieved stable serum 25-hydroxyvitamin D_3 (25D) levels between 16 and 117 nmol/L. At time of death, femora, L2 vertebrae and tibiae were processed for bone histomorphometric analyses and tibial cortical strength by 3-point mechanical testing. A significant association between serum 25D and trabecular bone occurred for both the distal femoral metaphysis ($R^2 = 0.34$, $P < 0.05$) and L2 vertebrae ($R^2 = 0.24$, $P < 0.05$). Tibia mid-shaft cortical bone was not, however, changed in terms of total volume, periosteal surface or endosteal surface as a function of vitamin D status. Furthermore, no changes to mechanical and intrinsic properties of the cortices were observed. We conclude that cortical bone is maintained under conditions of vitamin D deficiency in preference to cancellous bone in young growing rats.

1. Introduction

Although it has been well documented that vitamin D insufficiency, clinically defined by serum 25-hydroxyvitamin D_3 (25D) levels below 60 nmol/L is associated with increased fracture risk [1], the level of vitamin D required to maintain bone strength is controversial. The incidence of hip fracture in the elderly has been associated with decreased serum 25D levels rather than 1,25-dihydroxyvitamin D_3 (1,25D) levels suggesting that maintaining an adequate level of 25D is important for the prevention of osteoporosis [2]. Previously, we demonstrated trabecular bone loss in a rat model consistent with osteoporosis at moderately insufficient levels of 25D and osteomalacia at severe vitamin D deficiency [3]. Results from these finding suggest that while a circulating levels of 25D at 80 nmol/L is required to prevent the development of osteoporosis in rats, a positive linear relationship between serum 25D levels ranging between 20 and 115 nmol/L and femoral trabecular bone volume was demonstrated. Furthermore, these changes in trabecular bone volume were shown to be independent of changes to serum calcium, parathyroid hormone (PTH) and 1,25D levels [4].

While the effects of vitamin D deficiency on trabecular bone structure are well described, the effects on cortical bone volume and strength have not been reported in the vitamin D deficient normocalcaemic model. A number of studies using various rodent models have reported changes to bone strength with various effects on cortical and trabecular bone volume. For example, the effects of ovariectomy combined with a diet containing low levels of calcium in rats caused a significant reduction of femoral shaft strength with only a reduction in trabecular bone volume and no change in cortical bone volume [5]. In contrast, no changes in the strength or other biomechanical properties of the femoral shaft were observed in other vitamin D deficient or ovariectomised rats, despite showing a similar pattern of trabecular bone loss and maintenance of femoral and tibial cortical bone area in the mid-shaft [6]. Consistent with these findings, femoral neck strength was shown to be unchanged in aged ovariectomised rats which had significant loss of trabecular bone. However, no report was made of cortical bone volume in this study [7]. Furthermore, long-term ovariectomy studies in young rats were able to demonstrate maintenance [8] or gain [9] in

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cortical bone volume despite a rapid trabecular bone loss in the distal femoral metaphysis. Together these findings suggest that while characteristics of bone quality other than density and size may play important contributions to overall bone strength, the results may vary due to the conditions of hormone and calcium deprivation. The aim of the current study was to investigate the effects of vitamin D deficiency on cortical bone structure and strength in young male rats. We have utilised both micro-CT and the three-point bending test in our vitamin D deficient model of osteopenia to investigate tibial mid-shaft structure and strength.

2. Materials and methods

2.1. Animals

Twenty-four male Sprague–Dawley rats were raised in an incandescent-lighted environment, and were maintained on vitamin D deficient semi-synthetic diet containing 1% calcium and 0.6% phosphorus as previously published [4]. All animals were fed on their assigned diets from weanling until 10 weeks of age, at which point the animals were allocated to groups and pair-fed a modified AIN-93 diet (ICN Biomedicals, Aurora, OH, USA) containing 0.4% calcium and either 2, 4, 8 or 12 IU/day vitamin D (n = 5–6/group). The animals were fed their assigned diets until 30 weeks of age and sacrificed. Fasting blood were collected and femora, tibiae and lumbar spine were removed and processed for micro-CT analysis and 3-point mechanical strength testing at time of death. All animal procedures were approved by the Institute of Medical and Veterinary Science Animal Ethics Committee.

2.2. Biochemical analyses

Serum calcium was measured using a chemistry analyser (Trace Scientific reagents, Vic, Australia; Hitachi 911 automated analyser, Roche, IN, USA). Serum 1,25D and 25D were measured by a ^{125}I radioimmunoassay (RIA) (Immunodiagnostic Systems Ltd, Bolden, UK). Serum PTH was measured using rat-specific, two-site immunoradiometric assays (Immutopics, Inc., San Clemente, CA, USA).

2.3. Micro-computed tomography

The micro-architecture of the tibia was evaluated using a high resolution micro-CT system (Skyscan 1076, Brussels, Belgium) to obtain multiple X-ray transmission images. Transverse CT slices were acquired at the tibial mid-shaft using 18 μm slice increment. An 8 mm region of cortical bone, located 4 mm above and below the mid-point of the tibia was used for structural analyses. Periosteal surface area (mm^2) and endosteal surface area (mm^2) were assessed using CTan software (v1.7, Skyscan, Belgium). For 3D analysis, using the same technique as above in an 8 mm region of cortical bone, we assessed the total cross-sectional volume, cortical bone volume and medullary volume (TV, BV and MV, respectively, mm^3). Tibia length was measured using digital callipers.

2.4. Three-point mechanical strength testing

The mechanical properties of the tibiae were assessed by three-point bending method [10], performed by a miniature Instron materials testing machine (Instron 5848 MicroTester) with a 500 N load cell. Prior to testing, tibiae were thawed in PBS at room temperature for 30 min and mid-point of the tibia determined using digital callipers. The lower anvil points were set at 20 mm apart, equidistant from the mid-point for each bone. The upper anvil was lowered on to the mid-point at a rate of 1.0 mm/min for up to 5 mm maximum deflection in the bone or until the bone failed. Results were collected in Wavemaker (version 9.1.00, Instron, Instron Corp., Canton, MA, USA). From the force (F) versus displacement (D) curve we calculated, ultimate load to failure (ULF) and Young's modulus (E). E, defined as the intrinsic stiffness of the bone was based on the following calculation: $E = (F/D) \times (L3/48 \times I)$, where L = length of span and I = cross-sectional moment of inertia, derived using Image J (Java 1.6.0_10, USA). ULF was the maximum force required to break the bone. Breaking energy was obtained by calculating the area under the stress–strain curve, which defines the amount energy needed to cause a fracture. Yield point is a boundary above which stress causes permanent damage to the bone structure and is defined as the point when the stress–strain curve become non-linear.

Table 1
Serum levels of 25D, PTH and Ca in each dietary group. Values are mean (SEM).

	Dietary vitamin D (IU/day)			
	2	4	8	12
25D (nmol/L)	20.2[a] (1.6)	46.2[a] (4.4)	83.0[a] (3.0)	92.8[a] (8.0)
1,25D (pmol/L)	162.8 (13.2)	168.7 (14.1)	234.0[b] (30.6)	224.5[b] (16.4)
PTH (pmol/L)	6.0 (1.1)	6.2 (0.3)	6.6 (1.2)	5.1 (0.2)
Ca (mmol/L)	2.45 (0.01)	2.52 (0.02)	2.57 (0.07)	2.58 (0.09)

[a] $P < 0.05$ when compared with lower vitamin D treatment groups.
[b] Serum biochemistry from these animals are adapted from Ref. [2]. 25D, 25-hydroxyvitamin D; 1,25D, 1,25-dihydroxyvitamin D_3; PTH, parathyroid hormone; Ca, calcium.

2.5. Statistical analyses

One-way analysis of variance was used to analyse the effect of varying levels of dietary vitamin D on bone structure and strength. A value of $p < 0.05$ was considered to be statistically significant. Multi-linear regression analyses were performed on biochemical, morphological and biomechanical measures to determine interactions.

3. Results

3.1. Biochemistry

Stable levels of 25D were achieved by 18 weeks of age and were maintained until time of death at 30 weeks of age. Treatment groups achieved serum 25D levels ranging from 16 to 117 nmol/L with the highest in animals fed with 12 IU/day. There were no changes in serum PTH or serum Ca associated with varying vitamin D status (Table 1).

3.2. Bone structure and strength

Serum 25D levels were positively associated with both femoral metaphyseal trabecular bone (R^2 = 0.34, $P < 0.05$) and L2 vertebral bone (R^2 = 0.24, $P < 0.05$) (Fig. 1A and B). No changes to tibial length, cortical bone volume or measures of cortical bone distribution were observed between dietary vitamin D groups (Table 2). Furthermore,

Table 2
Body weights and bone structure measurements in the mid-shaft of the tibia. Values are mean (SEM).

	Dietary vitamin D (IU/day)			
	2	4	8	12
Tibia length (mm)	45 (0.4)	44 (0.4)	45 (0.3)	44 (0.6)
Cortical BV (mm^3)	44 (1.4)	44 (0.4)	47 (1.7)	46 (1.1)
Periosteal SA (mm^2)	121 (2.4)	120 (3.0)	124 (3.2)	122 (5.2)
Endosteal SA (mm^2)	55 (3.5)	50 (2.0)	62 (3.1)	52 (3.2)

BV, bone volume; SA, surface area.

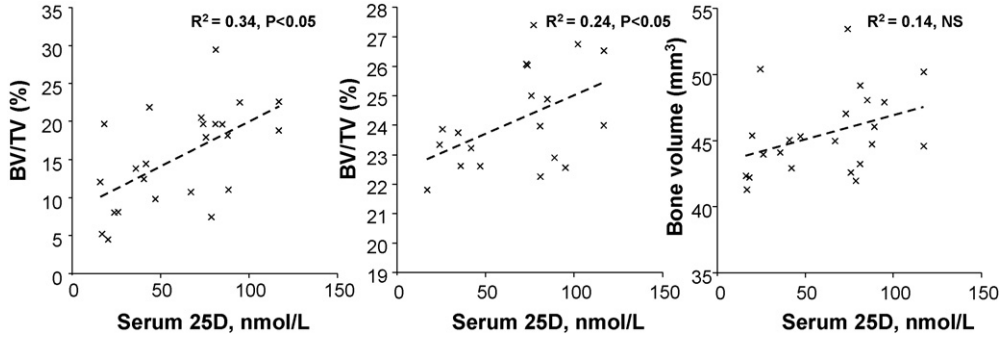

Fig. 1. The relationship between serum 25D and (A) femoral trabecular bone volume, (B) L2 vertebral trabecular bone volume and (C) mid-shaft tibial cortical bone volume in animals with serum 25D ranging between 16 and 117 nmol/L.

Table 3
Bone strength measurements in the tibial mid-shaft by 3-point mechanical testing. Values are mean (SEM).

	Dietary vitamin D (IU)			
	2	4	8	12
Ultimate load (N)	90 (4.8)	83 (2.4)	80 (3.3)	84 (4.6)
Yield point (N)	80.0 (4.7)	72.1 (2.1)	72.6 (3.1)	75.5 (5.6)
Breaking energy (mJ)	62.4 (6.9)	65.6 (7.6)	56.6 (2.8)	57.5 (13.1)
Young's modulus (GPa)	6.0 (0.6)	5.9 (0.1)	6.0 (0.6)	6.0 (0.4)

no statistically significant relationship occurred between serum 25D levels and mid-shaft tibial cortical bone volume ($R^2 = 0.24$, $P = 0.15$) (Fig. 1C), periosteal or endosteal surface area (data not shown). While cortical bone volume and distribution of bone was not altered due to vitamin D depletion, it was important to test the quality of the bone by 3-point mechanical testing. Cross-sectional moment of inertia (data not shown), ultimate load, yield point, breaking energy and Young's modulus were all unaffected by changes to dietary vitamin D (Table 3) or changes to serum 25D levels (data not shown).

4. Discussion

In contrast to our recent findings of vitamin D deficiency-related trabecular bone loss [4], our present findings indicate that tibial cortical bone volume and measures of cortical architecture were preserved even when growing rats were reduced to serum 25D levels as low as 16 nmol/L. This is consistent with previous studies, where trabecular bone loss due to other factors did not necessarily result in cortical bone loss [4,5,6]. Furthermore, others have demonstrated an absence of cortical bone loss [8] or some evidence of bone gain [9] during ovariectomy which occurs regardless of age and stage of bone growth. The findings suggest the stimulus for maintenance or increased cortical bone following ovariectomy may be an adaptive process in response to increasing mechanical forces on those structural elements that remain after initial bone loss. Thus, while we show marked effects of vitamin D depletion of trabecular bone loss in both the femur and vertebrae, the lack of effect of vitamin D depletion of cortical bone suggests that other factors such as mechanical strain determine the maintenance of its structure.

In addition to our observation that cortical bone volume did not change, we observed no redistribution of bone mineral. In particular, there was no change in either endosteal or periosteal surface indicating that overall bone turnover in this region was maintained at a constant level for all vitamin D diet groups. Cortical bone, which constitutes the majority of the skeleton has a predominantly mechanical function, while trabecular bone has both mechanical and metabolic functions as a store for calcium and phosphorus [11]. The ability of cortical bone to resist load, as a function of its geometry and intrinsic material properties is important. An important factor in the determination of bone structure and volume is mechanical strain. The application of mechanical forces results in the response of osteogenic/antiresorptive bone cells to maximise the load resisting properties [12]. The preservation of cortical bone structure and strength in growing rats in spite of the bone losing state of vitamin D deficiency highlights the dominance of mechanical forces.

Another possible explanation for the differential effect of vitamin D deficiency on cortical bone compared to trabecular bone is simply that cortical bone is less sensitive to conditions that lead to bone loss. The lower surface area to volume ratio inherent to cortical bone compared to the more complex trabecular architecture itself will contribute to the much slower changes seen in the cortical bone compare to the trabecular bone. An ovariectomy study in rats suggested that although indices of bone turnover were observed in the endocortical surface 1–2 months post-ovariectomy, significant reduction in cortical thickness in the femoral neck only occurred 1 year post-ovariectomy despite a significant change in the trabecular bone in the proximal tibial metaphysis as early as 14 days after ovariectomy [13]. The observation suggests loss of cortical bone may only be observed under more extreme bone loss conditions, such as marked calcium deprivation for an extended period of time or with ageing.

Although vitamin D depletion leads to a loss of trabecular bone, our present demonstration of preserved cortical bone volume, distribution and strength, indicates that the effect of vitamin D deficiency varies at least between the trabecular and cortical regions., Further investigations of the mechanisms that lead to trabecular bone loss while preserving cortical bone is warranted which may include the investigations into the distribution and activity of osteocytes in these bone compartments.

References

[1] D. Jesudason, A.G. Need, M. Horowitz, P.D. O'Loughlin, H.A. Morris, B.E. Nordin, Relationship between serum 25-hydroxyvitamin D and bone resorption markers in vitamin D insufficiency, Bone 31 (5) (2002) 626–630.
[2] M.C. Chapuy, M.E. Arlot, F. Duboeuf, J. Brun, B. Crouzet, S. Arnaud, P.D. Delmas, P.J. Meunier, Vitamin D3 and calcium to prevent hip fractures in the elderly women, N. Engl. J. Med. 327 (23) (1992) 1637–1642.
[3] P.H. Anderson, R.K. Sawyer, B.K. May, P.D. O'Loughlin, H.A. Morris, 25-Hydroxyvitamin D requirement for maintaining skeletal health utilising a Sprague–Dawley rat model, J. Steroid Biochem. Mol. Biol. 103 (3–5) (2007) 592–595.
[4] P.H. Anderson, R.K. Sawyer, A.J. Moore, B.K. May, P.D. O'Loughlin, H.A. Morris, Vitamin D depletion induces RANKL-mediated osteoclastogenesis and bone loss in a rodent model, J. Bone Miner. Res. 23 (11) (2008) 1789–1797.
[5] T.S. Kaastad, O. Reikeras, J.E. Madsen, S. Narum, J.H. Stromme, K.J. Obrant, L. Nordsletten, Effects of clodronate on cortical and trabecular bone in ovariectomized rats on a low calcium diet, Calcif. Tissue Int. 61 (2) (1997) 158–164.

[6] T.S. Kaastad, O. Reikeras, V. Halvorsen, J.A. Falch, K.J. Obrant, L. Nordsletten, Vitamin D deficiency and ovariectomy reduced the strength of the femoral neck in rats, Calcif. Tissue Int. 69 (2) (2001) 102–108.
[7] Z.Q. Peng, H.K. Vaananen, H.X. Zhang, J. Tuukkanen, Long-term effects of ovariectomy on the mechanical properties and chemical composition of rat bone, Bone 20 (3) (1997) 207–212.
[8] J.H. Waarsing, J.S. Day, J.A. Verhaar, A.G. Ederveen, H. Weinans, Bone loss dynamics result in trabecular alignment in aging and ovariectomized rats, J. Orthop. Res. 24 (5) (2006) 926–935.
[9] Y. Jiang, J. Zhao, H.K. Genant, J. Dequeker, P. Geusens, Long-term changes in bone mineral and biomechanical properties of vertebrae and femur in aging, dietary calcium restricted, and/or estrogen-deprived/-replaced rats, J. Bone Miner. Res. 12 (5) (1997) 820–831.
[10] C.H. Turner, D.B. Burr, Basic biomechanical measurements of bone: a tutorial, Bone 14 (4) (1993) 595–608.
[11] C.M. Bagi, N. Hanson, C. Andresen, R. Pero, R. Lariviere, C.H. Turner, A. Laib, The use of micro-CT to evaluate cortical bone geometry and strength in nude rats: correlation with mechanical testing, pQCT and DXA, Bone 38 (1) (2006) 136–144.
[12] J.R. Mosley, L.E. Lanyon, Growth rate rather than gender determines the size of the adaptive response of the growing skeleton to mechanical strain, Bone 30 (1) (2002) 314–319.
[13] M. Li, Y. Shen, T.J. Wronski, Time course of femoral neck osteopenia in ovariectomized rats, Bone 20 (1) (1997) 55–61.