Journal of Steroid Biochemistry & Molecular Biology 144 (2014) 159–162

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

Review

Adequate dietary vitamin D and calcium are both required to reduce bone turnover and increased bone mineral volume

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ARTICLE INFO

Article history:
Received 1 August 2013
Received in revised form
11 November 2013
Accepted 13 November 2013
Available online 2 December 2013

Keywords:
Vitamin D
Calcium
Bone turnover

ABSTRACT

Clinical studies indicate that the combination of vitamin D and dietary calcium supplementation is more effective for reducing fracture risk than either supplement alone. Our previous dietary studies demonstrated that an adequate serum 25-hydroxyvitamin D_3 (25D) of 80 nmol/L or more reduces bone RANKL expression, osteoclastogenesis and maintains the optimal levels of trabecular bone volume (BV/TV%) in young rats. The important clinical question of the interaction between vitamin D status, dietary calcium intake and age remains unclear. Hence, 9 month-old female Sprague-Dawley rats ($n = 5–6$/group) were pair-fed a semi-synthetic diet containing varying levels of vitamin D (0, 2, 12 or 20 IU/day) and dietary calcium (0.1% or 1%) for 6 months. At 15 months of age, animals were killed, for biochemical and skeletal analyses. While changes to serum 25D were determined by both dietary vitamin D and calcium levels, changes to serum 1,25-dihydroxyvitamin D_3 (1,25D) were consistently raised in animals fed 0.1% Ca regardless of dietary vitamin D or vitamin D status. Importantly, serum cross-laps levels were significantly increased in animals fed 0.1% Ca only when combined with 0 or 2 IUD/day of vitamin D, suggesting a contribution of both dietary calcium and vitamin D in determining bone resorption activity. Serum 25(OH)D_3 levels were positively correlated with both femoral mid-diaphyseal cortical bone volume ($R^2 = 0.24$, $P < 0.01$) and metaphyseal BV/TV% ($R^2 = 0.23$, $P < 0.01$, data not shown). In multiple linear regressions, serum 1,25$(OH)_2D_3$ levels were a negative determinant of CBV ($R^2 = 0.24$, $P < 0.01$) and were not a determinant of metaphyseal BV/TV% levels. These data support clinical data that reduced bone resorption and increased bone volume can only be achieved with adequate 25D levels in combination with high dietary calcium and low serum 1,25D levels.

This article is part of a Special Issue entitled '16th Vitamin D Workshop'.

Contents

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0960-0760/$ – see front matter.
http://dx.doi.org/10.1016/j.jsbmb.2013.11.009

1. Introduction

Osteoporosis is a preventable bone disease which can occur as a consequence of prolonged depletion of circulating levels of 25 hydroxyvitamin D (25D). The level of 25D required in humans to prevent osteoporosis has been reported to be approximately 75 nmol/L which is necessary to maintain normal bone mineral density and reduce the incidence of hip fracture in the elderly as determined by meta-analyses of clinical trials when vitamin D supplementation is 800 IU per day or greater in combination with calcium supplementation [1,2]. We have previously reported that vitamin D depletion in rats causes osteopenia in at least three skeletal sites including the distal and proximal femoral metaphyses and vertebrae. Furthermore, serum 25D levels ranging from 20 to 115 nmol/L were a positive and independent determinant of femoral trabecular bone volume [3]. However, the level of vitamin D required to prevent bone loss is likely to be dependent on factors such as age and nutrition and in particular the level of dietary calcium intake [4]. The co-administration of vitamin D and calcium reduces the incidence of hip fracture in elderly [5]. Numerous clinical trials demonstrate that vitamin D supplementation sufficient to raise serum 25D levels has greater efficacy in reducing fracture rates if combined with calcium supplementation [5,6]. It is clear that a diet containing high levels of calcium protects against bone loss by reducing both parathyroid hormone secretion and 1,25-dihydroxyvitamin D_3 (1,25D) production, which in turn reduces osteoclastic activity and anti-mineralisation [7,8]. We and others have reported that circulating levels of serum 25D are determined by both dietary vitamin D and calcium in both an animal model [9] and in postmenopausal women [10] suggesting that high dietary calcium may also play a role maintaining serum 25D levels.

In order to further understand the relationship between serum 25D levels and bone health, we have utilised our previously developed animal model [9] to address the question as to whether dietary calcium restriction influences the relationship between vitamin D status and bone mineral volume.

2. Materials and methods

2.1. Animals

Nine-month old female Sprague-Dawley rats ($n = 5$–6/group) were allocated to a diet containing varying levels of vitamin D_3 (0, 2, 12 or 20 IU/day) in combination with either 0.1% or 1% calcium, based on the recommended semi-synthetic diet for rodents (AIN-93-VX) [11]. All animals were maintained on this diet for 6 months, at which time animals were killed. All animal procedures were approved by the Institutional Animal Ethics Committee.

2.2. Biochemical analyses

Food was withdrawn from animals 16 h before blood samples were collected via the tail vein at time of death. Serum 1,25D and 25D levels were measured by RIA (Immunodiagnostic Systems Ltd., Bolden, UK). Levels of serum C-terminal telopeptide α1 chain of rat type I collagen (Cross-laps) were measured by EIA (Immunodiagnostic Systems Ltd., Bolden, UK). Serum calcium and inorganic phosphate, were measured using a chemistry analyser (Trace Scientific reagents, Vic, Australia; Hitachi 911 automated analyser, Roche, IN, USA).

2.3. Micro-computed topographical analyses

The proximal femur and tibia bone micro-architecture was analysed using a high-resolution micro-CT system (Skyscan 1072, Belgium) to obtain multiple X-ray transmission images. The resolution of scanning was 6.5 μm/pixel. Cross sectional images of the object were then reconstructed by a modified Feldkamp cone-beam algorithm, creating a complete 3D representation of internal microstructure and density. Adaptive thresholding technique was applied to reconstructed dataset prior to calculations of metaphyseal BV/TV%, Tb.N and Tb.Th. For cortical bone, an 8 mm mid-region of the femur was used to determine cortical bone volume.

2.4. Data expression and statistical analyses

The effects of diet on histological measurements and biochemical markers were statistically analysed by multivariate analysis of variance and Tukey's post hoc test analysis. Multiple linear regression analyses were used to identify determinants of bone mineral volume. A P value of 0.05 or less was considered to be statistically significant.

3. Results

3.1. Serum biochemistry

Serum 25D levels were lowest in the rats fed no vitamin D and 0.1% calcium diet (Table 1). Serum 25D levels were significantly greater in animals fed 1% calcium in the 0, 2 and 20 IU vitamin D_3/day fed animals when compared to animals fed 0.1% dietary calcium ($P < 0.05$). The animals fed 20 IU vitamin D3/day and 1% calcium recorded the highest mean serum 25D ($P < 0.05$). Despite the relative low serum 25D levels in animal fed 0 IU vitamin D_3/day and 0.1% calcium, serum 1,25D levels were significantly increased and comparable to levels in vitamin D-replete animals fed 0.1% calcium. Mean serum calcium levels were significantly elevated in the animals fed 20 IU vitamin D_3/day and 1% calcium. Serum cross-laps were highest in rats fed 0.1% Ca and receiving 0 or 2 ID vitamin D_3/day.

3.2. Bone mineral analyses

While all animals fed 1% calcium demonstrated higher metaphyseal BV/TV% of the distal femur, the highest BV/TV% was in rats fed either 12 or 20 IU vitamin D_3/day. The higher metaphyseal BV/TV% in 1% calcium fed rats was due to increased Tb.Th, except for the rats fed 20 IU vitamin D_3/day, where increased BV/TV% was due to both

Table 1
Serum levels of 25 hydroxyvitamin D, 1,25-dihydroxyvitamin D, calcium and cross-laps in rats fed varying levels of vitamin D and either 0.1% or 1% dietary calcium.

Vitamin D (IU/day)	0		2		12		20	
Dietary Ca (%)	0.1	1	0.1	1	0.1	1	0.1	1
Serum 25D (nmol/L) (sem)	21.7 (2.9)	84.5 (6.5)[a]	69.5 (9.4)	86.2 (7.8)	87.8 (10.7)	119.4 (11.6)[a]	90.9 (11.8)	161.3 (38.8)[a]
Serum 1,25D (pmol/L) (sem)	246.6 (30.2)	19.5 (6.5)[a]	226 (32.4)	48.0 (15.3)[a]	216.6 (31.8)	9.0 (4.5)[a]	248.6 (68.1)	13.8 (3.5)[a]
Serum Ca (mmol/L) (sem)	2.8 (0.1)	2.6 (0.1)	2.7 (0.2)	2.5 (0.1)	2.9 (0.2)	2.8 (0.2)	2.6 (0.2)	3.2 (0.2)[a]
Serum X-laps (nmol/L) (sem)	20.8 (2.4)	13.0 (1.5)[a]	21.7 (9.0)	6.0 (4.0)[a]	10.5 (2.1)	6.7 (2.2)	7.8 (2.0)	9.6 (5.1)

Values are mean (sem), $n = 5$–6. 25D: 25-hydroxyvitamin D_3; 1,25D: 1,25 dihydroxyvitamin D_3; Ca: calcium; X-laps, c-telopeptide fragments of collagen-type I.
[a] $P < 0.05$ vs. 0.1% Ca groups.

Table 2
Static bone histomorphometry of the distal femoral metaphysis.

Vitamin D (IU/kg)	0		2		12		20	
Dietary Ca (%)	0.1	1	0.1	1	0.1	1	0.1	1
BV/TV (%) (sem)	22.6 (1.8)	27.0 (1.8)[a]	26.3 (1.6)	26.9 (0.5)[a]	25.1 (0.9)	29.3 (1.0)[a]	25.8 (1.4)	29.3 (1.7)[a]
Tb.N (mm) (sem)	2.65 (0.1)	2.74 (0.12)	2.75 (0.12)	2.82 (0.05)	2.71 (0.13)	2.93 (0.07)	2.74 (0.11)	3.12 (0.11)[a]
Tb.Th (μm) (sem)	79.2 (3.1)	88.3 (1.5)[a]	82.7 (2.9)	87.0 (1.7)[a]	83.3 (2.1)	91.4 (2.3)[a]	86.6 (0.8)[b]	91.7 (2.5)[a]
Cort BV (mm^3) (sem)	27.2 (0.8)	28.6 (0.6)	27.3 (1.1)	28.8 (0.4)	27.3 (1.4)	30.8 (1.2)[a]	27.1 (0.9)	31.7 (1.2)[a]

Values are mean (sem), $n = 6$. BV/TV: bone volume/total volume; Tb.N: trabecular number; Tb.Th: trabecular thickness.
[a] $P < 0.05$ vs. 0.1% Ca groups.
[b] $P < 0.05$ vs. 0 IU Vit D/0.1% Ca.

Table 3
Dynamic bone histomorphometry of the distal femoral metaphysis.

Vitamin D (IU/kg)	0		2		12		20	
Dietary Ca (%)	0.1	1	0.1	1	0.1	1	0.1	1
MS/BS (sem)	29.5 (0.8)	32.4 (3.0)	32.4 (2.9)	33.7 (4.1)	33.8 (2.2)	25.7 (2.5)[a]	27.7 (1.8)	17.9 (1.7)[a]
MAR, (μm/day) (sem)	1.22 (0.05)	1.19 (0.06)	1.25 (0.04)	1.23 (0.01)	1.37 (0.04)	1.37 (0.04)	1.22 (0.02)	1.22 (0.06)
BFR ($\mu m^3/\mu m^2 \mu$day) (sem)	36.1 (1.6)	38.5 (3.8)	41.0 (4.7)	40.0 (1.3)	45.9 (2.3)	35.0 (3.1)[a]	33.9 (3.4)	21.8 (2.3)[a]
Oc/B.Pm, no. $10^{-3}\times$ (mm) (sem)	3.1 (0.7)	2.0 (0.4)	1.8 (0.2)[b]	1.4 (0.2)	2.1 (0.4)	1.4 (0.3)[a]	2.0 (0.2)	1.1 (0.1)[a]

Values are mean (sem), $n = 6$. MS/BS: mineralising surface/bone surface; MAR: mineral apposition rate; BFR: bone formation rate; Oc/B.Pm: osteoclast number/bone perimter.
[a] $P < 0.05$ vs. 0.1% Ca groups.
[b] $P < 0.05$ vs. 0 IU Vit D/0.1% Ca.

Table 4
Multiple linear regression equations for serum 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D as determinants of femoral cortical bone volume of the mid-diaphyseal region.

Independent variable	Equation	R^2	P value
Serum 25D + Serum 1,25D	CBV = +0.016 × 25D	0.24	0.005
	− 0.008 × 1,25D + 28.4	0.28	0.02
Multiple R^2		0.33	

CBV: cortical bone volume; 25D: 25 hydroxyvitamin D3; 1,25D: 1,25 dihydroxyvitamin D3.

Th.Th and Tb.N. Similar to BV/TV% measures, cortical bone volume was greatest in rats fed 12 and 20 IU vitamin D_3/day plus 1% Ca.

3.3. Relationships between biochemistry and bone structure

Serum 25D levels were positively correlated with both femoral mid-diaphyseal cortical bone volume ($R^2 = 0.24$, $P < 0.01$) (Table 4) and metaphyseal BV/TV% ($R^2 = 0.23$, $P < 0.01$, data not shown). In multiple linear regressions, serum 1,25D levels were a negative determinant of CBV ($R^2 = 0.24$, $P < 0.01$) (Table 4) and were not determinant of metaphyseal BV/TV% levels (Tables 2 and 3).

4. Discussion

The association between circulating 25D levels and bone mineral volume at two skeletal sites confirms our previous report that serum 25D levels are an independent predictor of bone mineral volume [3]. Previously, we reported that serum 25D levels were determined by both dietary vitamin D and calcium levels [9], suggesting that the benefits of high dietary calcium in improving bone mineral volume may be, in part, by preserving serum 25D levels. All animals fed the low 0.1% Ca diet demonstrated markedly increased serum 1,25D levels, which were a negative determinant of cortical bone volume, consistent with the reported roles for high circulating 1,25D levels in stimulating bone resorption and inhibition of mineralisation [12,13]. However, raised levels of both osteoclast numbers within the metaphysis and serum cross-laps occurred with higher serum 1,25D levels only when serum 25D levels were reduced, suggesting that 25D may also play a role in directly regulating the bone resorption response, as we have previously observed in in vitro studies [14,15]. Conversely, lower levels of mineralising surface and bone formation rate only occurred in those animals with levels of serum 25D above 90 nmol/L levels and with low 1,25D levels. This suggests that increased bone volume, associated with adequate vitamin D and dietary calcium, is due to reduced bone turnover which favours maintenance of bone mineral levels.

We have previously reported that 25D directly promotes bone mineralisation, through its conversion to 1,25D within bone cells [16,17]. It is plausible that vitamin D-depletion may directly impair local synthesis of 1,25D in bone cells and thus bone mineralisation. How local synthesis of 1,25D in bone cells contributes to changes in bone volume in the context of changes to circulating 1,25D remains to be fully elucidated. However, partial depletion of 25D, due to seasonal changes have been previously shown in human studies not to be associated with changes in intestinal calcium absorption [18], and yet both osteoid volume and mineralisation lag time were increased, highlighting that the association between serum 25D levels and bone mineral may be due to altered local synthesis of 1,25D [18].

Given the several reports of VDR-mediated activities in osteoblasts, osteocytes and osteoclast, as well as chondroblasts, which appear to regulate both anabolic and catabolic processes depending on the physiological context, data from this study support the notion that both 25D and 1,25D facilitate calcium flow in and out of the skeleton presumably to primarily preserve normcalcemia with both positive and negative effects on the skeleton.

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