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High dose dietary vitamin D_3 increases bone mass and strength in mice

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ABSTRACT

Vitamin D plays a critical role in skeletal homeostasis. Vitamin D supplementation is used worldwide to maintain optimal bone health, but the most appropriate level of supplementation remains controversial. This study aimed to determine the effects of varying doses of dietary vitamin D_3 on the mechanical properties and morphology of growing bone.

Eight-week-old female mice were supplied with one of 3 diets, each containing a different dose of vitamin D_3: 1000 IU/kg (control), 8000 IU/kg or 20,000 IU/kg. Mice had *ad libitum* access to the specialty diet for 4 weeks before they were culled and their tibiae collected for further analysis. The collected tibia underwent three-point bending and reference-point indentation from which their mechanical properties were determined, and cortical and trabecular morphology determined by micro computed tomography.

Dietary supplementation with 20,000 IU/kg vitamin D_3 resulted in greater ductility (~200%) and toughness (~150%) compared to the 1000 IU/kg control. The 20,000 IU/kg diet was also associated with significantly greater trabecular bone volume fraction and trabecular number. The 8000 IU/kg diet had no significant effect on trabecular bone mass.

We conclude that vitamin D_3 supplementation of 20,000 IU/kg during early adulthood leads to tougher bone that is more ductile and less brittle than that of mice supplied with standard levels of dietary vitamin D_3 (1000 IU/kg) or 8000 IU/kg. This suggests that dietary vitamin D_3 supplementation may increase bone health by improving bone material strength and supports the use of vitamin D_3 supplementation, during adolescence, for achieving a higher peak bone mass in adulthood and thereby preventing osteoporosis.

1. Introduction

It is generally established that vitamin D_3 is crucial for bone health through its actions as a regulator of minerals, and in turn, skeletal homeostasis in vertebrates (Anderson et al., 2011). Deficiencies in vitamin D_3 during childhood can have significant health consequences such as growth retardation (Rajakumar, 2003) and detrimental effects on bone mineral acquisition (Lehtonen-Veromaa et al., 2002) and bone remodelling (Outila et al., 2001; Cheng et al., 2003; Fares et al., 2003) leading to rickets (O'Riordan and Bijvoet, 2014). These consequences in adolescence are also a significant risk factor for the development of osteoporosis later in life (Dawson-Hughes et al., 1991; Lips, 2001).

The effects of vitamin D_3 as a treatment for osteoporosis in adulthood are controversial. Conflicting reports suggests vitamin D_3 supplementation in adulthood reduces (Bischoff-Ferrari et al., 2005; Tang et al., 2007), has no effect (Michaëlsson et al., 2003), or increases the incidence of osteoporotic fractures (Smith et al., 2007; Sanders et al., 2010). By comparing and normalising 23 separate studies, Reid et al. (2013) found that vitamin D supplementation was not effective in reducing fracture risk in those not experiencing vitamin D deficiency.

An alternative strategy for preventing osteoporosis is to optimise peak bone mass during growth via vitamin D_3 supplementation during adolescence. A number of intervention studies examining the effect of vitamin D_3 supplementation in adolescents have reported significant increases in bone mineral content (BMC) (El-Hajj Fuleihan et al., 2006; Viljakainen et al., 2006) and bone mineral density (BMD) (Du et al., 2004), while other studies have reported no beneficial effects (Andersen et al., 2008). Recent systematic reviews and meta-analysis (Winzenberg et al., 2010; Winzenberg et al., 2011) have concluded that vitamin D_3 supplementation during adolescence had no significant effect on BMC and BMD, however there has been no randomised controlled trial to assess the effect of vitamin D_3 supplementation on bone

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health. Currently the recommended daily intake of vitamin D_3 in infants, children and adolescents is 400 IU (Wagner and Greer, 2008). This dosage is based on clinical trials measuring biomarkers of vitamin D_3 status and the indirect observations that 400 IU of vitamin D_3 prevents and treats rickets (Wagner et al., 2006; Rajakumar and Thomas, 2005).

It is clear that there is a need for further investigation into the effect of vitamin D_3 dietary supplementation on bone mass in a model of growing bone where subjects are vitamin D_3 replete. Therefore, our objective was to determine whether increasing dietary vitamin D_3 levels (8000 and 20,000 IU/kg) above the standard levels (1000 IU/kg) significantly alters bone mass and strength in growing mice.

2. Methods and animals

2.1. Animals

7 week old female C57Bl/6 J mice were obtained from Monash Animal Services, Victoria and housed in a temperature and humidity controlled environment on a 12 h light/dark cycle. All animal work was approved by Victoria University Animal Ethics Committee. All animals were fed on standard growth diet (AIN-93G, containing 0.47% calcium (Ca), 0.35% phosphate (PO_4), vitamin D_3 1000 IU/kg) from weaning until 8 weeks of age, at which point the animals were randomly allocated to one of three diets for a period of 4 weeks. These diets were modified AIN-93G diets supplemented with set concentrations of cholecalciferol (vitamin D_3) as follows: control (1000 IU/kg, n = 10), 8000 IU/kg (n = 10) or 20,000 IU/kg (n = 7). The animals had ad libitum access to both food & water. All animals consumed the same amount of food (average of 2.5 g per day, no significant difference between groups). At 12 weeks of age, the mice were deeply anaesthetized with pentobarbital sodium (60 mg/kg injection i.p.), killed by cervical dislocation, and their tibiae harvested. Tibiae were carefully dissected free from soft tissues; lengths measured with a digital caliper, and stored at −80 °C prior to mechanical testing and further analysis.

2.2. Micro computed tomography (μCT)

Tibiae were analyzed by micro-computed tomography as described previously (Johnson et al., 2014) using the SkyScan 1076 System (Bruker-microCT, Kontich, Belgium). Images were acquired using the following settings: 9 μm voxel resolution, 0.5 mm aluminium filter, 44 kV voltage, and 220 μA current, 2300 ms exposure time, rotation 0.5°, frame averaging = 1. The images were reconstructed and analyzed using SkyScan Software programs NRecon (version 1.6.3.3), DataViewer (version 1.4.4), and CT Analyser (version 1.12.0.0) as previously described (Johnson et al., 2014).

CTAn software was then used to select the regions of interest for both the cortical (CTAn version 1.15.4.0) and trabecular (CTAn version 1.11.8.0) bone of each scan. Trabecular region of interest (ROI) was selected as a 2 mm region starting 0.5 mm below the proximal growth plate. Cortical ROI was selected as a 1 mm region starting 7 mm below the growth plate. The cortical ROI was chosen such that the middle of the ROI corresponded with the point at which load was applied to the bones in the three-point bending experiments.

The analysis of bone structure was completed using adaptive thresholding (mean of min and max values) in CT Analyser. The thresholds for analysis were determined based on multilevel Otsu thresholding of the entire data set, and were set at 45–255 for trabecular bone and 71–255 for cortical bone.

2.3. Three point bending

Each tibia was rehydrated overnight in phosphate buffered saline (PBS) at room temperature prior to testing. To determine the mechanical properties of cortical bone each tibia was loaded to failure at 0.5 mm/s using a Bose Biodynamic 5500 Test Instrument (Bose, DE, USA). The span between the lower supports was 10 mm. Prior to testing, the tibiae were kept moist in gauze swabs soaked in PBS. Bones were positioned such that the load was applied 8.75 mm from the top of distal condyle in the anterior-posterior (AP) direction with distal condyle facing downwards (Supplementary Fig. 1). Wintest software (WinTest 7) was used to collect the load-displacement data across 10 s with a sampling rate of 250 Hz. Structural properties including Ultimate force (F_U; N), yield force (F_Y; N), stiffness (S; N/mm), and energy (work) to failure (U; mJ) (Johnson et al., 2014) endured by the tibia were calculated from the load and displacement data as outlined in Jepsen et al. (2015). The yield point was determined from the load displacement curve at the point at which the curve deviated from linear. Widths of the cortical mid-shaft in the medio-lateral (ML) and antero-posterior (AP) directions, moment of inertia (I_{min}), and the average cortical thickness were determined by μCT in the cortical region described above. Tibial material properties, i.e., stress–strain curves were calculated from the structural properties (i.e., load-displacement curve) in combination with morphological data from μCT as outlined in Turner and Burr (1993). The obtained stress–strain curves reflect the stiffness, strength and failure properties of the bone material itself without the influence of geometry.

2.4. Reference-point indentation

Local bone material properties at the tibial mid-shaft were examined by reference point indentation (RPI) as previously described (Tang et al., 2007) using a BP2 probe assembly apparatus (Biodent Hfc, Active Life Scientific Inc., Santa Barbara, CA, USA). The BP2 assembly includes a 90-degree cono-spherical test probe with a ≤5 μm radius point and a flat bevel reference probe with ~5 mm cannula length and friction <0.1 N. Each sample was indented 5 times with the initial indentation occurring on the anterior surface of the bone, 6 mm from the tibia-fibula joint along the midline of the bone. Subsequent indentations were taken by moving the sample left, right, forwards or backwards approximately 1 mm in each direction from the initial indentation. The machine was used with the following settings; indentation force 2 N, 2 indentations per sec (Hz), 10 indentation cycles per measurement and touchdown force of 0.1 N. The distance the probe travels into the bone (total indentation distance [TDI]) is a measure of the bone's resistance to fracture; indentation distance increase (IDI) is the indentation distance in the last cycle relative to the first cycle and is correlated to bone tissue roughness; average unloading slope indicates the compressibility of the bone and can be used as a measure of stiffness (Johnson et al., 2014).

2.5. Statistics

All graphs are represented as the mean of all biological replicates. The number of animals (n) is reported on the graph or in the figure legends. All error bars are standard error of the mean. Significant differences were identified by one-way ANOVA with Tukey's post-hoc test (GraphPad Prism 6.0 software). Statistical significance was considered $p < 0.05$.

3. Results

3.1. Effects of vitamin D_3 dietary intervention on tibial structural properties

The highest level of dietary vitamin D_3 (20,000 IU/kg), but not the 8000 IU/kg dose was associated with a significantly greater (116%) failure displacement when compared to the control group (Fig. 1A). The 20,000 IU/kg diet was also associated with significantly greater post-yield displacement compared to the control (206%), and the 8000 IU/kg (154%) group (Fig. 1B). Tibiae from mice supplied with 20,000 IU/kg dietary vitamin D_3 also showed a significantly greater work-to-failure compared control tibiae (153%) (Fig. 1C). Varying dietary vitamin D_3 levels had no significant effect on ultimate load, ultimate