BRIEF COMMUNICATION:
RESEARCH REPORT

CURRENT DEVELOPMENTS IN NUTRITION

Vitamins and Minerals

Effect of Low Dietary Vitamin D Fed Prior to and During Pregnancy and Lactation on Maternal Bone Mineral Density, Structure, and Strength in C57BL/6 Mice

Christopher R Villa,[1] Sandra M Sacco,[2,3] Jianmin Chen,[1] Amel Taibi,[1] Elena M Comelli,[1,2,4] and Wendy E Ward[1,2,3]

[1]Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada; [2]Department of Kinesiology, Faculty of Applied Health Sciences, Brock University, St. Catharines, Ontario, Canada; [3]Center for Bone and Muscle Health, Faculty of Applied Health Sciences, Brock University, St. Catharines, Ontario, Canada; and [4]Joannah and Brian Lawson Centre for Child Nutrition, Faculty of Medicine, University of Toronto, Toronto, Ontario, Canada

ABSTRACT

Several studies have shown that diets containing lower vitamin D than in the AIN-93G diet do not compromise bone structure, bone mineral density (BMD), and/or bone strength in male and female mice. This study determined if a diet containing low vitamin D from prepregnancy through to the end of lactation maintained these bone outcomes to a similar extent as a high vitamin D diet. Mice were fed an AIN-93G diet with 25 (LD diet) or 5000 (HD diet) IU vitamin D/kg diet from premating through to lactation ($n = 15$/group). Of the major structure outcomes, only cortical area fraction of the distal femur was lower ($P < 0.05$) with the LD diet. Lumbar vertebra BMD was lower ($P < 0.05$) with LD whereas distal femur BMD and bone strength at 3 sites did not differ. Dams fed an LD diet premating through to the end of lactation had largely similar bone outcomes to dams fed a HD diet. *Curr Dev Nutr* 2021;5:nzab114.

Keywords: AIN-93 diets, bone microarchitecture, fracture, micro-computed tomography, calcium, vitamin D

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CRV and SMS are joint first authors.

Address correspondence to WEW (e-mail: wward@brocku.ca).

Abbreviations used: BMC, bone mineral content; BMD, bone mineral density; BV, bone volume; BV/TV, bone volume fraction; Conn.D, connectivity density; Ct.Ar, cortical bone area; Ct.Ar/Tt.Ar, cortical area fraction; Ct.Th, cortical thickness; DA, degree of anisotropy; DXA, dual energy X-ray absorptiometry; Ecc, mean eccentricity; Ec.Pm, endocortical perimeter; HD, high vitamin D diet; HFS, high fat-high sucrose; L3, the third lumbar vertebra; LD, low vitamin D diet; Ma.Ar, marrow area; Ps.Pm, periosteal perimeter; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; Tt.Ar, total cross-sectional area inside the periosteal envelope; TV, total volume; $25(OH)D_3$, 25-hydroxyvitamin D_3.

Introduction

Understanding vitamin D requirements for bone health in rodent models is complex. Furthermore, some studies suggest that the current concentration of vitamin D in the widely used AIN-93 reference diet (1) may be present in excess of actual requirements to support bone health throughout the lifespan. Maternal and postweaning exposure to low versus high concentrations of dietary vitamin D (25 versus 5000 IU/kg diet) altered some structural outcomes of trabecular bone in C57BL/6 male (2) but not female (3) sibling offspring, whereas cortical bone and bone mineral density (BMD) were unaffected in both sexes. Of note, offspring from these studies were challenged with an obesogenic diet (high in fat and sucrose, HFS) to exacerbate effects on bone health from the age of 15 d through to 7 mo. Dietary calcium was maintained at the usual concentration in the AIN-93G diet (0.5%). Also, C57BL/6 male mice fed a vitamin-D-deficient diet (0 IU vitamin D/kg diet) from the age of 10 mo through to 24 mo had similar bone structure to mice fed a standard concentration of vitamin D in AIN-93 diets (1000 IU vitamin D/kg diet) (4). Together, these findings suggest that the concentration of dietary vitamin D in the AIN-93 diets is in excess of the requirement for a healthy skeleton. Importantly, this suggests that the AIN-93G diet may mask the effect of a novel dietary intervention targeting bone.

The present study adds to existing findings by focusing on the effect of low dietary vitamin D during pregnancy and lactation – a time when bone is mobilized to provide substantive amounts of calcium to the fetus and subsequently via maternal milk to facilitate skeletal development (5). Changes in BMD as well as strength and structure during pregnancy and lactation in mice have been extensively reviewed (5). However, a low concentration of vitamin D in the context of the AIN-93G diet has not been thoroughly studied during this life stage although it is a diet of choice for studies investigating effects of a dietary intervention even during pregnancy and lactation (6). The study objective was

to determine if a maternal diet low in vitamin D, and previously shown to modestly impact a few outcomes of bone structure in their male offspring and not bone strength in their male or female offspring, was sufficient to maintain bone health of dams who had undergone pregnancy and lactation.

Methods

Animals, diet, and tissue collection

The description of the animal protocol and breeding has been described in previous publications in which male and female offspring outcomes were reported (2, 3). For this study, female C57BL/6J mice aged 3 wk ($n = 30$) were purchased from Jackson Laboratories. This sample size was for convenience given the main outcomes for the larger study pertained to offspring outcomes previously reported (2, 3). Mice were randomly assigned to a modified AIN-93G diet (Diet # TD.119290, Dyets Inc.) that contained 0.5% calcium with either: *1*) 5000 IU (High, HD) vitamin D_3/kg diet or *2*) 25 IU (Low, LD) vitamin D_3/kg diet. For reference, the AIN-93G diet contains 1000 IU vitamin D_3/kg diet (7). At the age of 7 wk, pregnant dams were housed individually while staying on their respective diets. Because the objective of the main study was to determine if exposure to high dietary vitamin D from conception through to weaning positively programs systemic inflammation along with bone health in male and female offspring fed an obesogenic diet (2, 3), a HFS diet (44.2% fat and 19.8% sucrose by kcal), with its respective high or low vitamin D concentration, was introduced at day 15 of lactation as mice started to consume a solid diet in addition to dam milk (Diet# TD.120612 for high vitamin D, TD.120613 for low vitamin D, Harlan Laboratories) (2, 3). All micronutrients, excluding vitamin D, were adjusted proportionately for the higher energy provided in the HFS diet. Dams were killed at day 21 of lactation (LD, $n = 14$; HD, $n = 13$) by carbon dioxide asphyxiation followed by cervical dislocation (3 dams were killed earlier as they did not become pregnant and therefore were not included in the study). Serum was collected along with the right femur and the third lumbar vertebra (L3) that were cleaned of soft tissue, wrapped in saline-soaked gauze, and stored at −80°C. Covance Laboratories Inc. confirmed the dietary vitamin D concentrations provided to dams, as previously reported (2) using LC-MS/MS analysis. The study received ethical approval from the local animal care committee at the University of Toronto (Protocol Number: 20009576).

Serum 25-hydroxyvitamin D

Serum 25-hydroxyvitamin D ($25(OH)D_3$) was measured using LC-MS/MS at the end of lactation (day 21 of lactation) at the Analytical Facility for Bioactive Molecules of the Centre for the Study of Complex Childhood Diseases, The Hospital for Sick Children (Toronto, Ontario, Canada).

Micro-CT to measure bone structure of femur and L3

Three skeletal sites were studied: trabecular bone at the right distal femur and L3, and cortical bone at the femur diaphysis using micro-CT (Skyscan 1176, BrukerCT) as previously reported (3). Trabecular bone outcome measures included: bone volume (BV, mm^3), total volume (TV, mm^3), bone volume fraction (BV/TV, %), trabecular thickness (Tb.Th, mm), trabecular number (Tb.N, mm^{-1}), trabecular separation (Tb.Sp, mm), degree of anisotropy (DA, no unit), and connectivity density (Conn.Dn, $1/mm^3$). Specific to cortical analyses, the shrink-wrap function was applied to stretch over holes that were larger than 30 pixels in diameter. Cortical bone measures included: total cross-sectional area inside the periosteal envelope (Tt.Ar, mm^2), cortical bone area (Ct.Ar, mm^2), cortical area fraction (Ct.Ar/Tt.Ar, %), average cortical thickness (Ct.Th, mm), periosteal perimeter (Ps.Pm, mm), endocortical perimeter (Ec.Pm, mm), marrow area (Ma.Ar, mm^2), and eccentricity (Ecc, no unit).

Bone mineral content and density and biomechanical strength of the femur and L3

The right femur and L3 were scanned in air using DXA (Orthometrix; Host Software version 3.9.4 and Scanner Software version 1.2.0) to determine the bone mineral content (BMC) and BMD of the whole femur, its proximal 1/3 region rich in trabecular bone, and of L3 as previously described (3). Biomechanical strength testing was performed at 3 sites (femur neck, femur midpoint, L3) using a materials testing system (Model 4442 Universal Testing System, Instron Corp.) (3).

Statistical analysis

Statistical analyses were performed using SigmaStat (Jandel Scientific). Results are expressed as mean ± SEM. Students t-test was used to compare the outcomes between the LD and HD groups. Statistical significance was defined as $P < 0.05$.

Results

Body weights

Body weights of dams fed LD and HD were similar at the time of mating (LD = 17.6 ± 0.2 g, HD = 17.4 ± 0.3 g, $P > 0.05$) and at the end of lactation (LD = 25.8 ± 0.5 g, HD = 25.4 ± 0.5 g, $P > 0.05$).

Serum $25(OH)D_3$

Serum $25(OH)D_3$ concentration at the end of lactation was significantly different between groups ($P < 0.001$) and reflected the low (3.3 ± 0.2 nmol/L, $n = 3$/group) and high (67.1 ± 3.7 nmol/L, $n = 3$/group) dietary concentrations of vitamin D.

Bone structure

Representative images of trabecular (distal femur, L3) and cortical (femur diaphysis) bone structure for LD and HD groups are shown in **Figure 1**. At the distal femur, there were no significant differences in trabecular bone structure (**Table 1**). At the femur diaphysis, a site rich in cortical bone, significantly higher Ct.Ar/Tt.Ar was observed in mice fed HD compared with those fed LD (Table 1). In addition, significantly lower Ma.Ar and Ec.Pm were observed in mice fed HD compared with those fed LD, whereas there were no statistically significant differences in Ct.Ar, Tt.Ar, Ct.Th, Ps.Pm, or Ecc observed between the 2 groups. For L3, there were no differences in trabecular bone properties observed between the LD and HD groups (Table 1).

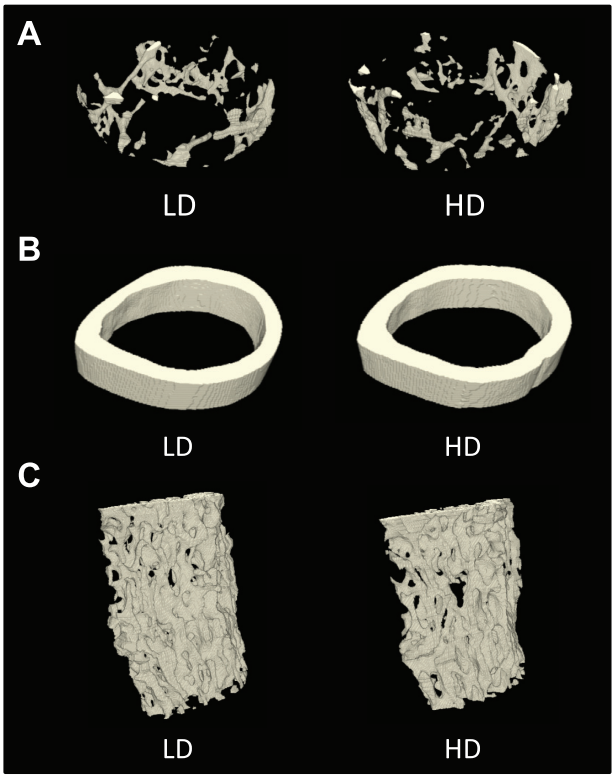

FIGURE 1 Representative images of A) trabecular bone at the distal femur; B) cortical bone at the distal femur; and C) trabecular bone at L3 for a mouse fed LD or HD. Representative images were selected based on mean values for BV/TV at distal femur and L3, and Ct.Ar/Tt.Ar at distal femur. BV/TV, bone volume fraction; Ct.Ar/Tt.Ar, cortical area fraction; HD, high vitamin D diet; LD, low vitamin D diet.

BMC, BMD, and biomechanical strength of the femur and L3

There were no differences in BMC or BMD at the whole or proximal third femur between the LD and HD groups (Table 1). In addition, peak load at the femur midpoint and at the femur neck did not differ between LD and HD groups. At L3, BMD was significantly higher ($P = 0.028$) in the HD group compared with the LD group, whereas no differences in BMC or peak load were observed (Table 1).

TABLE 1 Bone mineral, trabecular and cortical bone structure, and peak load at multiple skeletal sites of dams fed low (LD) or high (HD) vitamin D at the end of lactation

	Low vitamin D (LD)	High vitamin D (HD)
Bone mineral		
Whole femur		
BMC, mg	16.89 ± 0.33	17.58 ± 0.55
BMD, mg/cm^2	44.70 ± 0.64	46.93 ± 1.07
Proximal 1/3 femur		
BMC, mg	6.54 ± 0.10	6.81 ± 0.22
BMD, mg/cm^2	49.63 ± 0.63	51.78 ± 1.25
L3		
BMC, mg	16.41 ± 0.60	17.41 ± 0.65
BMD, mg/cm^2	46.98 ± 0.71	50.23 ± 1.18[1]
Trabecular bone structure		
Distal femur		
TV, mm^3	2.095 ± 0.030	2.089 ± 0.055
BV, mm^3	0.095 ± 0.007	0.103 ± 0.009
BV/TV, %	4.517 ± 0.301	4.907 ± 0.396
Tb.Th, mm	0.058 ± 0.001	0.057 ± 0.001
Tb.N, 1/mm	0.772 ± 0.045	0.856 ± 0.058
Tb.Sp, mm	0.538 ± 0.023	0.490 ± 0.028
DA, no unit	1.559 ± 0.030	1.696 ± 0.063
Conn.Dn, 1/mm^3	15.8 ± 1.6	19.3 ± 2.1
L3		
TV, mm^3	2.418 ± 0.156	2.460 ± 0.125
BV, mm^3	0.449 ± 0.019	0.484 ± 0.037
BV/TV, %	19.098 ± 0.983	19.561 ± 0.864
Tb.Th, mm	0.060 ± 0.002	0.060 ± 0.001
Tb.N, 1/mm	3.158 ± 0.0814	3.225 ± 0.102
Tb.Sp, mm	0.210 ± 0.003	0.206 ± 0.004
DA, no unit	2.029 ± 0.046	2.057 ± 0.012
Conn.D, 1/mm^3	132.647 ± 5.856	134.298 ± 8.015
Cortical bone structure		
Distal femur		
Tt.Ar, mm^2	1.923 ± 0.021	1.903 ± 0.022
Ct.Ar, mm^2	0.713 ± 0.006	0.754 ± 0.018
Ct.Ar/Tt.Ar, %	37.2 ± 0.5	39.6 ± 1.0[1]
Ct.Th, mm^2	0.147 ± 0.002	0.158 ± 0.004
Ma.Ar, mm^2	1.209 ± 0.022	1.148 ± 0.025[1]
Ps.Pm, mm	5.374 ± 0.031	5.332 ± 0.035
Ec.Pm, mm	4.350 ± 0.042	4.210 ± 0.051[1]
Ecc, no unit	0.694 ± 0.005	0.682 ± 0.013
Bone strength		
Femur midpoint		
Peak load, N	11.51 ± 0.31	13.05 ± 0.79
Femur neck		
Peak load, N	10.91 ± 0.76	10.23 ± 0.78
L3		
Peak load, N	36.89 ± 1.67	39.16 ± 2.62

[1]Denotes statistical significance ($P < 0.05$) versus low dose (LD).
Data are expressed as mean ± SEM, n = 12–13 for BMC and BMD, n = 12/group for bone structure analyses, n = 11–14 for bone strength.
BMC, bone mineral content; BMD, bone mineral density; BV, bone volume; BV/TV, bone volume fraction; Conn.D, connectivity density; Ct.Ar, cortical bone area; Ct.Ar/Tt.Ar, cortical area fraction; Ct.Th, cortical thickness; DA, degree of anisotropy; Ecc, mean eccentricity; Ec.Pm, endocortical perimeter; Ma.Ar, marrow area; Ps.Pm, periosteal perimeter, Tt.Ar, total cross sectional area inside the periosteal envelope; Tb.N, trabecular number; Tb.Sp, trabecular separation; TV, total volume; Tb.Th, trabecular thickness.

Discussion

Although the serum 25(OH)D_3 concentration was significantly lower at the end of lactation among mice fed LD from age 3 wk onwards compared with mice fed HD, the majority of outcomes of bone mineral quantity and bone structure – and at multiple skeletal sites – did not differ between dams fed low versus high concentrations of dietary vitamin D. Of note was that the structure at cortical rather than trabecular bone sites was where some differences were observed. However, lower cortical bone fraction in the LD group did not result in weaker bone strength at the femur midpoint or femur neck suggesting this structural change did not have a functional detriment.

That adequate calcium can mask the effect of vitamin D deficiency on bone has been known for many decades. In classic experiments, vitamin-D-deficient rats that received infusions of calcium and phosphorus to maintain serum calcium concentrations were shown to have normal bone development (8, 9). These and subsequent studies, including some from our group (10), suggest that vitamin D does not

have a direct effect on bone, but rather it is the decreased availability of calcium that leads to impaired mineralization of bone. Specifically, offspring of these dams who continued to be fed their respective diets after weaning showed minimal detriments to bone at the age of 7 mo (2, 3). These findings in male and female offspring suggest that calcium may have compensated for low dietary vitamin D intakes in mice. A more recent study that did not feed an obesogenic diet, showed that lowering both vitamin D and calcium, to a concentration of 100 IU and 0.25%, respectively, does not compromise BMD or bone structure in female mice when feeding these diets from weaning through to the age of 4 mo, representing young adulthood (10). This suggests that both the concentration of vitamin D and calcium in AIN-93G may be in excess for bone health at this life stage whereas effects at older ages have not yet been studied.

Major strengths of the study are the comprehensive set of outcomes to measure the quantity (bone mineral) and quality (structure, strength) of bone, and that these outcomes were measured at multiple skeletal sites representing different amounts of cortical and trabecular bone. A potential limitation of the study is the exclusion of a diet with the concentration of vitamin D in the control diet (1000 IU/kg) and thus a more detailed assessment of bone outcomes in terms of a dose response. Although the comparison with a group with a 5-fold higher concentration of vitamin D arguably provides greater confidence of the lack of problems at this lower level of dietary vitamin D. Other potential limitations include the single endpoint measure for bone outcomes such that the BMD, bone structure and strength immediately pre- and post-pregnancy is not known. Also, a follow-up study with an a priori sample size calculation could more conclusively support the findings of the present study. Due to limited sample, only 25(OH)D_3 was measured in the serum though calcitriol, parathyroid hormone, and other bone markers were not assessed. These aspects can be followed up in a future study.

In conclusion, dams fed LD from weaning through to the end of lactation have largely similar bone structure compared with dams receiving HD. Given the similarity in bone structure between groups despite the challenge of pregnancy and lactation, suggests that when dietary calcium is not a limiting factor, low vitamin D in the AIN-93G diet exceeds that needed to support BMD, bone structure, and bone strength during this life stage. In a recent Issues and Opinions in *The Journal of Nutrition*, Klurfeld et al. highlighted several concerns regarding the AIN-93 rodent diet formulas in terms of dietary fiber, the carbohydrate and fat components, as well as the resultant higher body weight (11). Thus, findings from the present study and others (2–4) suggest that consideration of bone-supporting nutrients such as vitamin D – and likely also calcium (10, 12) – should be part of the conversation regarding a potential revision of these diets. A control diet that contains a concentration of vitamin D in excess of the actual need for a mouse during pregnancy and lactation may mask the effect of either lowering the concentration of dietary vitamin D or of another dietary intervention aimed at modulating bone health in such a mouse model, and thus lead to a misinformed conclusion.

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Data Availability

Data in the manuscript will be made available upon reasonable request.

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