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

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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 pre-pregnancy 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 pre-pregnancy 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 pre-pregnancy 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

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 preg-
nancy 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 per-
tained to offspring outcomes previously reported (2, 3). Mice were ran-
domly 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 D3/kg diet or (2) 25 IU (Low, LD) vitamin D3/kg diet. For ref-
ence, the AIN-93G diet contains 1000 IU vitamin D3/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 re-
spective 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 pro-
vided in the HFS diet. Dams were killed at day 21 of lactation (LD, n = 14; HD, n = 13) by carbon dioxide asphyxiation followed by cer-
vical dislocation (3 dams were killed earlier as they did not become
pregnant and therefore were not included in the study). Serum was col-
lected 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
d concentrations provided to dams, as previously reported (2) using LC-
MS/MS analysis. The study received ethical approval from the local an-
imal care committee at the University of Toronto (Protocol Number:
20009576).

Serum 25-hydroxyvitamin D
Serum 25-hydroxyvitamin D (25(OH)D3) 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 fe-
mur 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, mm3), total vol-
ume (TV, mm3), 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 den-
sity (Conn.Dn, 1/mm3). 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, mm2), cortical bone area (Ct.Ar,
mm2), cortical area fraction (Ct.Ar/Tt.Ar, %), average cortical thickness
(Ct.Th, mm), periosteal perimeter (Ps.Pm, mm), endocortical perime-
ter (Ec.Pm, mm), marrow area (Ma.Ar, mm2), 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 de-
termine the bone mineral content (BMC) and BMD of the whole fe-
mur, its proximal 1/3 region rich in trabecular bone, and of L3 as previ-
ously 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 com-
pare the outcomes between the LD and HD groups. Statistical signifi-
cance 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)D3
Serum 25(OH)D3 concentration at the end of lactation was signifi-
cantly 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 be-
 tween the LD and HD groups (Table 1).
There were no differences in BMC or BMD at the whole or proximal 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.

**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.

**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

| Bone mineral | Low vitamin D (LD) | High vitamin D (HD) |
|--------------|------------------|---------------------|
| 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        |
| Trabecular bone structure |       |                     |
| Distal femur |                   |                     |
| TV, mm$^3$    | 2.095 ± 0.30    | 2.089 ± 0.055      |
| BV, mm$^2$    | 0.095 ± 0.007   | 0.103 ± 0.009      |
| BV/TV, %      | 4.517 ± 0.301   | 4.907 ± 0.396      |
| Tb.Th, mm     | 0.085 ± 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$^2$    | 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.Dn, 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      |
| 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.

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)D₃ 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, Kluifeld 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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The authors’ contributions were as follows—EMC and WEW: designed the research; CRV, JC, and AT: conducted the in vivo trial; CRV and SMS: were responsible for tissue analyses and data collection; SMS: analyzed data and performed statistical analyses; SMS and WEW: wrote the manuscript with all authors providing a critical review of the content along with feedback; WEW: had primary responsibility for final content; and all authors: read and approved the final manuscript.

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

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