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Bone development in growing female mice fed calcium and vitamin D at lower levels than is present in the AIN-93G reference diet

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ABSTRACT

Background: The AIN-93G reference (REF) diet is used to allow the comparison within and between studies of different research groups but its levels of vitamin D (vit D) and calcium (Ca) may be higher than required for healthy bone structure and bone mineral density (BMD).
Objective: To determine if lower dietary levels of Ca (3.5, 3 or 2.5 g Ca/kg diet) at 1 of 2 levels of vit D (100 or 400 IU/kg diet) supports similar development of bone structure and BMD compared to AIN-93G reference (REF) diet in female CD-1 mice at 2 and 4 months of age.
Methods: Within a trial, weanling female mice (n = 12–15/group) were randomized to 1 of 4 diets until necropsy at 4 months of age: Trial 1: 100 IU vit D/kg + 3.5, 3 or 2.5 g Ca/kg diet or 1000 IU vit D/kg + 5 g Ca/kg diet (REF); and Trial 2: 400 IU vit D/kg + 3.5, 3 or 2.5 g Ca/kg diet or 1000 IU vit D/kg + 5 g/kg diet (REF). At age 2 and 4 months, *in vivo* bone structure and BMD were assessed using micro-computed tomography (μCT) at the proximal and midpoint tibia. At age 4 months, lumbar vertebra 4 (L4) and mandible structure were analyzed *ex vivo*, femur strength at midpoint and neck was assessed and serum 25(OH)D_3 and PTH were quantified.
Results: For Trial 1 (100 IU vit D/kg), there were no differences in tibia structure at age 2 and 4 months nor L4 or mandible structure or femur strength at the midpoint or neck at 4 months of age despite lower serum 25(OH)D_3 among all groups compared to REF. For Trial 2 (400 IU vit D/kg), mice fed 2.5 g Ca/kg diet had lower ($p < 0.05$) Ct.Ar/Tt.Ar and Ct.Th at the tibia midpoint compared to REF. Furthermore, Ct.Th. was greater in REF and 3.5 g Ca/kg diet compared to 2.5 g Ca/kg diet at age 2 but not 4 months of age. At L4, BV/TV was lower ($p < 0.05$) in the 3 g Ca/kg diet group compared to REF at age 4 months. There were no differences among groups for serum 25(OH)D_3 or femur strength at the midpoint or neck. Serum PTH was not elevated compared to REF in either Trial.
Conclusion: Lowering both dietary vit D (100 IU/kg) and Ca (2.5 g/kg) in AIN-93G diet did not result in differences in bone development of female CD-1 mice at early adulthood. Translational relevance of bone studies conducted using the AIN-93G diet may be affected by its high vit D and Ca content.

1. Introduction

Rodent models are commonly used to investigate the effects of early life diet on bone mineral density (BMD) and bone structure, with the long term goal of developing dietary strategies to optimize bone development in humans (Ward et al., 2016; Kaludjerovic and Ward, 2010; Fischer et al., 2017; Halloran et al., 2010). When investigating the potential beneficial effects of novel foods or food components, using a consistent reference diet such as the AIN-93G diet (Reeves, 1989, 1997; Reeves et al., 1993a, 1993b), reduces the variation when comparing findings within and between laboratories and ensures that observed effects are in fact due to the dietary intervention and not due to a

Abbreviations: ANOVA, analysis of variance; BMC, bone mineral content; BMD, bone mineral density; BV/TV, percent bone volume; Ca, calcium; Conn.Dn, connectivity density; Ct.Ar/Tt.Ar, cortical area fraction; Ct.Th, cortical thickness; DA, degree anisotropy; Ecc, mean eccentricity; Ec.Pm, endocortical perimeter; ISO, isoflavones; L4, lumbar vertebrae 4; Ma.Ar, medullary area; PBM, peak bone mass; Ps.Pm, periosteum perimeter; PTH, parathyroid hormone; P, phosphorus; REF, AIN-93G reference diet; ROI, region of interest; SEM, standard error mean; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Th, trabecular thickness; vit D, vitamin D; μCT, micro computed tomography

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variation in the base diet.

The approach of using early life diet to set a trajectory for a long-term health outcome is generally referred to as 'nutritional programming'. We have previously demonstrated that there is a window of opportunity during early life for novel food components, such as soy isoflavones, to favourably program bone outcomes at early adulthood in terms of higher BMD and improved bone structure and bone strength in female mice (Kaludjerovic and Ward, 2010; Dinsdale et al., 2012; Kaludjerovic and Ward, 2009; Kaludjerovic and Ward, 2015). While these studies have used the AIN-93G reference diet that is recommended for supporting growth, pregnancy and lactation, the levels of vitamin D (vit D, 1000 IU/kg) and calcium (Ca, 5 g/kg) in this diet may be higher than required for normal bone development, measured as BMD and bone structure in mice and rats (Glenn et al., 2014; Villa et al., 2016; Hunt et al., 2008). Findings from our group have demonstrated that normal bone development, measured as BMD and biomechanical bone strength, occurs with a significantly lower level of vit D (25 IU/kg) in mice fed an obesogenic diet, in inflammatory prone female mice or in healthy male mice (Glenn et al., 2014; Villa et al., 2016; Jahani et al., 2014). In these studies, dietary Ca was kept constant at 5 g/kg and diets were fed from weaning until 3 (Glenn et al., 2014; Jahani et al., 2014) or 7 months of age (Villa et al., 2016). In growing female Sprague-Dawley rats, Ca levels were manipulated by examining both lower and higher levels than in the AIN-93G reference diet, 1 through 7 g/kg. Vit D was kept constant at 1000 IU/kg. BMD, biomechanical bone strength and bone structure were assessed at the end of the 13 week feeding trial. Bone development was reported to be healthy if Ca intakes met or exceeded 2.5 g/kg (Hunt et al., 2008). This level is lower than the requirement established by the National Research Council of 5 g Ca/kg diet, the level of Ca in the AIN-93G diet (National Research Council, Committee on Animal Nutrition, Board on Agriculture, 1995).

While the aforementioned studies have altered the level of vit D or Ca, the effect of lowering both the level of dietary vit D and Ca in combination on BMD and bone structure has not been thoroughly studied. Previous literature has demonstrated that no effect on bone health occurs when Ca is lowered to 2.5 g/kg diet, at REF vit D level, (Hunt et al., 2008) and no differences in serum Ca and PTH occurs in the absence of vit D and 4 g Ca/kg diet (Anderson et al., 2007). By reducing both the levels of vit D and Ca, it is possible to avoid or attenuate compensatory mechanisms that may mask the effect of either low vit D or Ca when the other nutrient is provided in excess. Moreover, the implication of providing higher than required levels of vit D or Ca is that the potential positive effects of a dietary intervention to support healthy bone development may be diminished. Thus, a benefit of a dietary intervention to bone development could be masked due to a potential excess of vit D or Ca in the diet. Also of consideration is that humans are often not consuming these nutrients at recommended levels (Health Canada, 2009) and thus using a rodent diet that does not provide these nutrients in excess levels for bone health, may be more appropriate for extrapolating findings to humans.

When this study was designed, there was a paucity of studies in which both Ca and vit D were lowered in combination, so we used literature in which either Ca *or* vit D were lowered to guide our decisions as to which levels of vit D and Ca to study in combination. We had previously used a vit D level of 25 IU/kg diet in the CD-1 mouse model and shown that there was no detriment to BMD or bone strength (Jahani et al., 2014) but another study had shown that femur BMC and BMD were reduced at 25 IU/kg diet but not at 100 IU/kg diet compared to the level in REF diet (1000 IU/kg diet) (Fleet et al., 2008). Thus, we chose to study 100 IU as a level of vit D that was markedly lower than the REF diet but would likely support bone development. A level of 400 IU vit D/kg diet was selected as femur BMC and BMD were previously shown to be similar to mice fed REF diet (1000 IU vit D and 5 g Ca/kg diet) (Fleet et al., 2008) and we wanted to include a level of vit D that was in between the REF level and the 100 IU vit D/kg diet. Within each of these levels of vit D (100 IU and 400 IU/kg diet), three different levels of Ca were provided. Based on the finding that dietary levels of Ca below 2.5 g Ca/kg diet compromised BMD, strength and structure of femurs in rats (Hunt et al., 2008), this level was studied as the lowest level of dietary *Ca*. But because this level of Ca had been studied in the context of a regular level of vit D and in rats, we chose to study two additional and slightly higher levels of Ca (3 and 3.5 g Ca/kg diet), in case the level of 2.5 g Ca/kg diet was insufficient to support healthy, normal bone development in mice.

The objective of this study was to determine if lower levels of vit D and Ca compared to the AIN-93G diet supports normal bone development at 2 and 4 months of age in female mice. Bone structure and BMD at the proximal and midpoint of the tibia were measured using *in vivo* micro-computed tomography (μCT) to assess longitudinal bone development within each mouse. Structure at other skeletal sites such as lumbar vertebra 4 (L4) and mandible were measured *ex vivo* at 4 months of age. Bone strength was examined at the midpoint and neck of the femur. Based on previous studies, we hypothesized that all of the combinations of Ca and vit D that were tested would support normal bone development as BMD, structure and/or strength was not compromised when Ca was higher than 2.5 g/kg diet or dietary vit D was 100 IU/kg diet or higher.

2. Methods

2.1. Animals and diets

This study was conducted in accordance with the Canadian Council of Animal Care and all experimental procedures have been approved by the Animal Care Committee at Brock University, St. Catharines, Canada. A total of twenty-two timed-pregnant CD-1 mice were purchased from Charles River Laboratories (St. Constant, QC, Canada) and fed AIN-93G diet (REF) and water *ad libitum* throughout pregnancy and lactation. Mice were housed under standard environmental conditions (12 h light:12 h dark cycle, room temperature of 23 °C). Considering that vit D can be endogenously produced with exposure to ultraviolet beta radiation, LED lighting with zero ultraviolet emissions were used in the mouse room.

Weanling female mice (21 days of age) (n = 118) were randomized to 1 of 4 color-coded diets (within each of the two trials) until necropsy at 4 months of age: Trial 1: AIN-93G REF diet containing 1000 IU vit D/kg + 5 g Ca/kg diet (REF) (TD.94045) or 1 of 3 experimental diets containing 100 IU vit D/kg + 3.5 (TD.160266), 3 (TD.160267), or 2.5 g Ca/kg diet (TD.160268) and Trial 2: AIN-93G REF diet containing 1000 IU vit D/kg + 5 g Ca/kg diet (REF) (TD.94045) or 1 of 3 experimental diets containing 400 IU vit D/kg + 3.5 (TD.160269), 3 (TD.160270), or 2.5 g Ca/kg diet (TD.160271). All diets were prepared by Envigo, Madison, WI. The level of Ca and vit D in the diets were confirmed by a third party (Maxxam Analytics, Mississauga, ON, Canada) (Table S1). Vit D was in the form of vitamin D_3. Ca was included as calcium phosphate, monobasic, monohydrate (6.4 g calcium phosphate, monobasic, monohydrate/kg diet for all the diets) and as calcium carbonate (6.1 g calcium carbonate/kg diet for diet containing 3.5 g Ca/kg diet; 4.86 g calcium carbonate for diet containing 3 g Ca/kg diet; and 3.6 g calcium carbonate for 2.5 g Ca/kg diet).

Throughout the study, mice were housed 4 to 5 per cage, body weight was measured once weekly and food intake was measured 2 to 3 times each week. Food intake per mouse per day was calculated by dividing the total food consumed by the number of mice in a cage per day.

The right tibia of the offspring was scanned at 2 and 4 months of age using high resolution *in vivo* μCT (SkyScan 1176, Bruker microCT, Belgium) while mice were anesthetized with isoflurane. At 4 months of age, and immediately prior to euthanasia, blood samples were collected after a 12 h fast for serum 25(OH)D_3 and PTH analyses. Immediately after euthanasia, the lumbar vertebrae 4 (L4), right mandible and