Reduction of ovariectomy-induced bone loss in rats by menaquinone-4, cholecalciferol, and (n-3) polyunsaturated fats

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Abstract

Menopause and ovariectomization both lead to a rapid loss of bone mineralization. Conflicting reports exist concerning the ability of menaquinone-4, a form of vitamin K routinely used in Japan, to successfully ameliorate the bone mineral loss associated with overiectomization in rats. We hypothesized that menaquinone-4 may be acting in conjunction with other nutrients, specifically vitamin D$_3$ or (n-3) polyunsaturated fatty acids (PUFA), to affect bone mineral loss in the ovariectomized state. Two experiments were performed using 6-month-old ovariectomized rats placed on diets containing normal or supraphysiological levels of menaquinone-4 while also varying the dietary content of vitamin D$_3$ or (n-3) PUFA for eight months. Femur bone mineral density was monitored by DXA. Additionally, femur bone mineral ash and femur bone strength was measured in the second experiment. Supraphysiological levels of menaquinone-4 with either twice the recommended level of Vitamin D$_3$ or a high n-3/n-6 ratio in the diet significantly reduced bone mineral loss in ovariectomized rats after eight months of treatment. Additionally, rats fed either a high n-3/n-6 ratio diet or supraphysiological levels of menaquinone-4 for eight months maintained bone strength measures compared to ovariectomized control animals. Supraphysiological levels of menaquinone-4 did ameliorate the bone mineral loss associated with ovariectomization when used in conjunction with vitamin D or (n-3) PUFA.

Introduction

For more than seventy years vitamin K has received wide recognition for its metabolic function in hemostasis. More recently interest in the vitamin has focused on its role in bone metabolism. A recent meta-analysis has provided evidence that vitamin K reduces the incidence of osteoporotic fractures [1]. Vitamin K functions as a cofactor in the posttranslational gamma-carboxylation of glutamic acid residues in at least three known vitamin K-dependent bone proteins: osteocalcin (OC), matrix Gla protein (MGP), and Protein S [2, 3]. The growing interest in vitamin K is driven by evidence that it may improve bone quality and attenuate rapid bone loss characteristic of osteoporosis. This interest is further fueled by efforts to address the projected increase in incidence of osteoporotic fractures as life expectancy increases and the baby boomer generation ages [4, 5]. Inadequate dietary vitamin K intake [6, 7] or coumarin-related anticoagulant therapy [8] has been associated with an increased risk of hip fracture and low bone mineral density. The current dietary recommendation for vitamin K (1 mg/kg body weight) is in question in light of optimal bone health [9, 10]. Confounding this issue is the apparent need to consider age and gender with respect to dietary recommendations [11]. It appears that menaquinone-4 (menatetrenone or MK4), and not phylloquinone (vitamin K1) may be responsible for the vitamin’s metabolic effect on bone metabolism [12-14]. Although MK4 is not common to most Western diets it is synthesized in the body from dietary phylloquinone [15]. There is some argument whether the observed inhibitory effect of the vitamin on bone loss is due in large measure to the aliphatic side chain (geranylgeraniol) of the vitamin [16] or MK4 as a whole [17]. Multiple studies have examined the effect of MK4 in the ovariectomized (OVX) rat model of postmenopausal osteoporosis. The findings have been inconsistent [18-20]. This study attempted to address these discrepancies and examine the effect of supraphysiological MK4 supplementation on bone quality in OVX rats. A further purpose of this research was to study the effect of MK4 in combination with either cholecalciferol (vitamin D3, VD3) or (n-3) polyunsaturated fatty acids (PUFA).

Methods

Animal research. Both experiments in this study were approved by the Brigham Young University Institutional Animal Care and Use Committee (IACUC), completed in full compliance with all committee guidelines, and monitored by the University Veterinarian. Twenty-five-week-old Simonsen Albino
adult female rats were purchased from Simonsen Laboratories (Gilroy, CA). Ovariectomy and sham surgeries were performed by Simonsen Laboratories prior to shipping. Animals were individually housed in suspended wire-bottom cages to reduce coprophagy and the resulting menaquinone intake. Distilled water was made available ad libitum. The animals were housed in a secured room maintained at 26°C with a twelve-hour light/dark cycle.

Diets. Both experiments used a semi-purified AIN-76A vitamin K-deficient diet patterned after Harlan Teklad diet TD 81053 [15] with the exception that the soy protein was not alcohol-water extracted as this option was no longer available. An AIN-76 vitamin K-deficient vitamin mix (Harlan Teklad #227940, Madison, WI) was used in the preparation. The diets were prepared once each month and stored frozen in plastic freezer bags at -80°C to prevent microbial menaquinone production. Ovariectomized animals were pair-fed to the SHAM group in order to decrease the effect of ovariectomy-induced weight gain. Ovariectomized animals were stratified according to body weight, and randomly assigned a dietary treatment. In the first experiment VD3 (cholecalciferol; Sigma-Aldrich, Inc., St. Louis, MO) and MK4 (menaquinone-4; RIA International, LLC., Whippany, NJ) were supplemented to the diets in varying amounts. VD3 and MK4 were mixed into corn oil (Mazola brand) before adding the oil to the diet. The experiment had five groups of rats, seven animals per group: a SHAM group and an OVX control group were fed the same diet, while the remaining three OVX groups (n=7) in the experiment received the experimental diets. A control corn-oil based diet patterned after Harlan Teklad TD81053 vitamin K-deficient diet was given to the SHAM-operated control and OVX control groups. MK4 was added to the control diets to meet vitamin K requirement (1 mg MK4/kg diet). The remaining three OVX groups (n=7) in the first experiment were supplemented as follows: [1] OVX n-3 PUFA (Menhaden fish oil-based, (n-6)/(n-3) ratio=1:5; MK4, 1 mg/kg diet); [2] OVX MK4 (corn oil-based, (n-6)/(n-3) ratio=56:1; MK4, 1500 mg/kg diet); and [3] OVX n-3 PUFA+MK4 (Menhaden fish oil-based, (n-6)/(n-3) ratio=1:5; MK4, 1500 mg/kg diet).

Bone Densitometry. Both experiments included dual energy x-ray absorptiometry (DXA) bone densitometry scans [22] of the right femur at baseline and every subsequent two months (2, 4, 6, and 8 months) using a GE Lunar Prodigy Advance densitometer (Madison, WI) in the small animal analysis mode. Animals were anesthetized with ketamine (37.5 mg/kg body weight) and domitor (0.5 mg/kg body weight). Following DXA scanning, antisedan (0.5 mg/kg body weight) was administered as a sedation-reversal agent for domitor.

Animals were randomized and duplicate scans were collected for each animal at each time point for experiment 1 and five scans were collected for each animal at each time point in experiment 2 with the high and low values ignored. Bone mineral density was analyzed in two regions of interest (ROI) (Illustration 1). The first ROI was defined as an eight millimeter square region positioned at the distal femur as described by Binkley, et al. [20]. The second ROI enclosed the femoral diaphysis and the aforementioned eight millimeter distal ROI.

Bone Mineral Ash. In the second experiment, total femur bone mineral ash was measured ex vivo following mechanical testing in the second experiment. Fractured femurs were dried at 105°C overnight and ashed at 750°C for twelve hours in porcelain crucibles. Animals were randomized and duplicate scans were collected on the excised femur for second experiment investigating (n-3) PUFA and MK4. Animals were sacrificed one week following the DXA scan at eight months. The right femur was disarticulated and soft tissue left intact. Femurs were wrapped in isotonic saline-soaked gauze, sealed individually in plastic bags and stored at -20°C. The femurs were removed from the freezer and thawed at room temperature overnight before mechanical testing. Soft tissue was removed and the femur was wrapped again in saline-soaked gauze until just prior to testing. The three-point bending test was adapted from ANSI/ASAE S459 [23] using an Instron Series 3340 Load Frame (Norwood, MA). Briefly, the support span fulcra for the test were adjusted to obtain a support length to femur (middiaphysis) diameter ratio greater than 10:1 and spanning from metaphysis to metaphysis. Femurs were broken in a randomized order. A loading force was applied to the middiaphyseal anterior surface over a single-cycle
ramp function at a constant loading rate of 10 mm/min. The force-deformation curve was collected until the load dropped by forty percent of the peak flexure load. The middiaphyseal bone cross section was modeled as a hollow ellipse to calculate the moment of inertia by: moment of inertia = \( \frac{p}{64} \left[ \left( B^2-D^3 \right) - \left( b^2-d^3 \right) \right] \) (Illustration 2). Ultimate bending strengths were calculated from the peak flexure load by: ultimate bending strength = \( \frac{\text{peak flexure load} \times \text{support span} \times \text{middiaphyseal radius}}{4^2 \times \text{moment of inertia}} \). 

Yield stress (the stress at which the bone reacts plastically rather than elastically) was calculated from the flexure load at yield (offset 0.2 percent), the load at which permanent deformation occurs. The apparent modulus of elasticity was calculated by: modulus of elasticity = \( \frac{\text{peak flexure load} \times \text{(support span)}}{4^2 \times \text{moment of inertia}} \). All force measurements are reported in megapascal (MPa).

Results and Discussion

Animal Weight. Experiment 1 animal weights for the treatment groups after 8 months of treatment were (g, mean ± SD) 313.5 ± 58.6, 363.6 ± 36.6, 359.7 ± 19.4, 362.9 ± 28.1, and 362.7 ± 45.9 for the Sham, OVX, OVX VD3, OVX MK4, and OVX VD3+MK4 groups respectively. There was no significant difference between treatment group weight means at eight months (ANOVA p > 0.05). In experiment 2, average animal weights for the treatment groups after 8 months of treatment were (g, mean ± SD) 299.2 ± 31.2, 349.4 ± 19.0, 355.1 ± 22.6, 345.3 ± 20.5, 356.2 ± 13.5 for the Sham, OVX, OVX n-3, OVX MK4, and OVX n-3+MK4 groups respectively. There was a significant difference between treatment group average weight at 8 months (ANOVA p < 0.0001) with the Sham treatment group average being different from the other treatments (Tukey-Kramer post-hoc analysis p < 0.001).

Percent change BMD (Experiment 1). The SHAM control confirmed ovariectomy-induced bone loss. No significant difference was observed in the percent change in BMD for distal femur in vivo between the OVX control and any of the three OVX supplemented groups through 8 months of treatment (Illustration 4A). However, a significant (p < 0.05) difference was observed in the percent change in BMD for the diaphyseal femur in vivo between the OVX control and the OVX VD3+MK4 group at 8 months (Illustration 4B). The combination of VD3 and MK4 on diaphyseal femur BMD demonstrated an additive effect.

DISCUSSION

Ovariectomy results in a loss of mineral from the bone as observed in these studies by comparing bone property values from the OVX control groups to the SHAM group which retained the ovaries. Supraphysiological MK4 has been reported to protect against OVX-induced decreases in bone strength after six months of supplementation in rats [18, 19]. In contrast, it has also been reported that neither supraphysiological phylloquinone nor MK4 reduce OVX-induced bone turnover or preserve bone strength after three months [20]. This study attempted to reconcile the discrepancy regarding the effect of supraphysiological vitamin K, namely MK4, on ovariectomy-induced bone loss. Another objective of this study was to describe the effect of MK4 in combination with either VD3 or (n-3) PUFA on bone quality.

The first experiment examined the effect of VD3 (twice the dietary recommendation), in combination with MK4 on BMD. Neither vitamin alone had any significant effect on bone retention, but the combination of vitamins significantly inhibited OVX-induced bone loss after eight months of supplementation as determined by percent change in BMD over a period of eight months. The effect of increasing both vitamins appeared to be additive. Recently it has been reported that phylloquinone in combination with vitamin D and calcium supplementation exhibited a synergistic effect on bone mineral content of healthy, nonosteoporotic women [26]. The combined effect of the MK4 and VD3 may be a result of steroid and xenobiotic receptor crosstalk. There is evidence that MK4 functions as a hormone in bone homeostasis by binding the SXR steroid and xenobiotic receptor and inducing...
MK4 regulated the transcription of several bone-specific genes in osteosarcoma cells, including bone alkaline phosphatase (ALP), osteoprotegerin (OPG), osteopontin (OPN), and matrix Gla protein (MGP). Each of these proteins is a marker of osteoblastic activity. Osteoprotegerin binds the osteoblastic membrane protein RANKL and obstructs it from binding its cognate receptor RANK found on the osteoclast precursor cell membrane, resulting in the inhibition of osteoclastogenesis [28]. In addition, ALP, OPG, OPN and MGP mRNA expression are all increased by vitamin D [27, 29, 30]. A second mechanism has been demonstrated by which the activated SXR receptor inhibited the VDR-activation of CYP24 promoter [31]. CYP24 enzymes are responsible for the catabolism of the active 1,25(OH)2D3 metabolite [32]. Via the SXR steroid and xenobiotic receptor, MK4 may not only activate osteoblastic genes, but prolong the presence of active VD3.

The second experiment in this study examined the effect of Menhaden fish oil, high in (n-3) PUFA, and Menhaden oil in combination with MK4 on bone mineralization and bone strength. Fish oil is established as an excellent source of omega-3 fats, such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Neither (n-3) PUFA or MK4 alone had any significant effect on BMD, but each significantly preserved bone strength and stiffness. The combination of (n-3) PUFA and MK4 increased bone mineral retention as measured by bone ash, an observation not seen by DXA, presumably due to the variation in measurement values and small sample size.

At present (n-3) PUFAs are believed to attenuate bone loss in part by inhibiting the production of inflammatory lipid prostaglandin E2 (PGE2) which stimulates bone resorption [33]. PGE2 is a metabolite of the (n-6) PUFA arachidonic acid (AA). This reaction is catalyzed by cyclooxygenase-2 (COX-2). The COX-2 enzyme is also responsible for the conversion of EPA into prostaglandin E3 (PGE3). PGE3 stimulates bone resorption with similar efficiency as PGE2, but EPA is a less efficient precursor for bone resorbing prostaglandins than AA [34]. COX-2 has greater specificity for AA than EPA and thus favors PGE2 synthesis rather than PGE3 [35]. Fish oil-based diets increase the concentration of EPA, which then competes with AA as substrates for the COX-2 enzyme. The dietary ratio of (n-6)/(n-3) PUFA therefore may modulate the rate of bone resorption. Both PGE2 and PGE3 induce COX-2 mRNA expression [36]. Interestingly, high concentrations of EPA have been shown to reduce the expression of COX-2 [37, 38]. It is necessary to note that a high-dosage EPA supplementation increased bone resorption in ovariectomized rats [39]. MK4 as well has been shown to attenuate bone resorption by inhibiting PGE2 synthesis [40]. More recently there is evidence which suggest that the DHA component of fish oil is responsible for optimizing bone quality [39, 41]. Although not significant, it was observed that (n-3) PUFA and MK4 together appear to exhibit a detrimental effect on bone strength qualities. Any significant effect of either nutrient alone was negated when combined with the other nutrient. Considering both nutrients reduce bone resorption this was an entirely unanticipated effect. There appears to be no literature addressing this novel observation, and any potential mechanism. Fish oil has been reported to inhibit gamma-glutamylcarboxylase, and consequently vitamin K-dependent clotting factors in male rats [42]. Gamma-carboxylated MGP has been shown to interact with growth factors to prevent soft tissue calcification [43, 44]. Fish oil may antagonize the vitamin K-dependent carboxylation of MGP, and might be evident with an increase in soft-tissue calcification. This potential antagonism requires further investigation.

In summary, this study demonstrated that supraphysiological MK4 in conjunction with either VD3 or (n-3) PUFA supplementation decelerates ovariectomy-induced bone loss in the diaphyseal femur of Simonsen Albino rats. In addition, this research showed that either supraphysiological doses of MK4 or high (n-3) PUFA supplementation preserves the bending strength and stiffness of the femur in ovariectomized rats.

Literature Cited


Illustrations

Illustration 1

DXA analyzed regions. 1) Eight millimeter square region of distal femur. 2) femoral diaphysis.

Illustration 2

The middiaphyseal femur cross section as a hollow ellipse. Image adapted from Lopez and Markel [24].
Illustration 3

Force displacement curve. Adapted from Lopez and Markel [24].

Illustration 4

Experiment 1: % change from initial BMD by DXA. A) Distal femur. B) Diaphyseal femur.
Illustration 5

Experiment 2: 8-month percent change from initial BMD by DXA A) Distal Femur. B) Diaphyseal femur.

Illustration 6

Experiment 2: femur bone ash.
Illustration 7

Experiment 2: femur yield bending strength.

Illustration 8

Experiment 2: femur ultimate bending strength.
Illustration 9

Experiment 2: femur modulus of elasticity.
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