Differential Effect of Vitamin K and Vitamin D Supplementation on Bone Mass in Young Rats Fed Normal or Low Calcium Diet

Jun Iwamoto¹, Tsuyoshi Takeda¹, Shoichi Ichimura², Yoshihiro Sato³, and James K. Yeh⁴

¹Department of Sports Medicine, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan; ²Department of Orthopaedic Surgery, Kyorin University School of Medicine, Tokyo, Japan; ³Department of Neurology, Mitate Hospital, Fukuoka, Japan; ⁴Metabolism Laboratory, Department of Medicine, Winthrop-University Hospital, NY, USA.

The purpose of this study was to clarify the differential effect of vitamin K and vitamin D supplementation on bone mass in young rats fed a normal or low calcium diet. Ninety female Sprague-Dawley rats, 6 weeks of age, were randomized by stratified weight method into nine groups with 10 rats in each group: baseline control, and 0.5% (normal) or 0.1% (low) calcium diet, either alone, or with vitamin K (30 mg/100g, food intake), vitamin D (25 μg/100g, food intake), or vitamin K + vitamin D. After 10 weeks of feeding, bone histomorphometric analyses were performed on cortical bone of the tibial shaft and cancellous bone of the proximal tibia. Vitamin K supplementation increased the maturation-related cancellous bone gain and retarded the reduction in the maturation-related cortical bone gain in rats fed a low calcium diet, and increased the maturation-related cortical bone gain in rats fed a normal calcium diet. Vitamin D supplementation reduced the maturation-related cancellous bone gain, prevented the reduction in periosteal bone gain, and enhanced the enlargement of the marrow cavity, with no significant effect on the reduction in the maturation-related cortical bone gain in rats fed a low calcium diet, and increased the maturation-related cancellous and cortical bone gains with increased periosteal bone gain in rats fed a normal calcium diet. An additive effect of vitamin K and vitamin D on the maturation-related cortical bone gain was found in rats fed a normal calcium diet. This study shows the differential effects of vitamin K and vitamin D supplementation on cancellous and cortical bone mass in young rats fed a normal or low calcium diet, as well as the additive effect on cortical bone under calcium sufficient condition.

Key Words: Calcium, vitamin K, vitamin D, bone growth, combination therapy

INTRODUCTION

A maximal peak bone mass at skeletal maturity is considered the best protection against age-related bone loss and subsequent fracture risk.¹,² Adequate calcium intake during growth influences peak bone mass. However, adolescent girls and young adult women are the most likely to have calcium-deficient diets, hindering the attainment of peak bone mass.

It is known that both vitamin K and vitamin D affect bone metabolism. In particular, vitamin D increases intestinal calcium absorption via the action of 1,25 (OH)₂ vitamin D₃,⁵ while vitamin K increases renal calcium reabsorption.⁶,⁷ Thus, supplementation of these vitamins may help to increase peak bone mass in adolescent girls. Furthermore, because an additive effect of vitamin K and vitamin D supplementation on bone mass has been demonstrated in adult ovariectomized rats, young rats, and postmenopausal women with osteoporosis,⁵,⁸,¹⁰ it would be expected that vitamin K and vitamin D supplementation might act on bone additively in adolescent girls. However, the effects of vitamin K and/or vitamin D supplementation on cortical and cancellous bone growth have not been well studied.
The purposes of this study were, firstly, to clarify the differential effect of vitamin K and vitamin D supplementation on cancellous and cortical bone mass in young rats fed a normal or low calcium diet by bone mineral density (BMD) measurement and static bone histomorphometric analysis, and secondly, to determine whether combined supplementation of vitamin K and vitamin D would have an additive effect on cancellous and cortical bone mass.

MATERIALS AND METHODS

Treatment of animals

Ninety female Sprague-Dawley rats, 4 weeks of age, were purchased from Hilltop Lab. Animals, Inc. (Scottsdale, PA, U.S.A.). They were fed a pelleted standard chow diet containing 0.5% calcium, 11.25µg/100g vitamin D, and 3mg/100g vitamin K (Rodent Laboratory;Ralston Purina Co., St. Louis, MO, U.S.A.). Rats were housed under local vivarium conditions (temperature 23.8°C and 12h on/off light cycle) with free access to water and chow diet. After two weeks of adaptation to their new environment, the rats, 6 weeks of age, were randomized by stratified weight method into nine groups of 10 rats in each group: baseline control (BLC) group, 0.5% (normal) or 0.1% (low) calcium diet (NC or LC, respectively) group, NC or LC + vitamin K (30mg/100g, food intake) (NCK or LCK, respectively) group, NC or LC + vitamin D (25µg/100g, food intake) (NCD or LCD, respectively) group, and NC or LC + vitamin K + vitamin D (NCKD or LCKD, respectively) group. These special synthetic diets were formulated by and purchased from Harlan Teklad (Madison, WI, U.S.A.). The supplemented vitamin K (menatetrenone) was supported by the Eisai Pharmaceutical Co., Ltd. (Tokyo, Japan). The doses of vitamin K and vitamin D used in their supplementation groups were not physiologic but pharmacologic to test the beneficial effect of supplementation of these vitamins on bones, and may be extremely higher than human doses. The body weight of the rats was monitored weekly, and the experimental period was 10 weeks. This study was carried out at Winthrop-University Hospital. Rats were maintained according to the National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals, and the animal protocols were approved by the Laboratory Animal Care Committee of Winthrop-University Hospital.

Preparation of specimens

The rats in the BLC group were sacrificed at 6 weeks of age. The rats in the experimental groups except the BLC group were sacrificed at 10 weeks after the start of the experiment. They were anesthetized with 80mg/kg ketamine injected intraperitoneally together with 12mg/kg xylazine, and killed by exsanguination. Upon death, serum was collected from all rats. The right femur and tibia were removed. The femur was used for measurements of bone length, wet weight, bone volume (BV), and bone density, and was then used for the measurement of BMD by dual energy X-ray absorptiometry (DXA). The tibia was processed for static bone histomorphometric analysis. The tibia was fixed in cold 70% ethanol overnight, and was then cut into three parts using an Isomet saw (Buehler, Lake Bluff, IL, U.S.A.). The proximal tibia and tibial shaft with fibular junction were stained with Villanueva Osteochrome Bone Stain (Polysciences, Warrington, PA, U.S.A.) for 5 days. The specimens were dehydrated sequentially in ascending concentrations of ethanol (70%, 95%, and 100%) and xylene and then embedded in methyl methacrylate (EM Science, Gibbstown, NJ, U.S.A.) at 4°C according to the method of Erben. Cross-sections of the tibial shaft just proximal to the tibia-fibular junction were cut at 40-µm thickness using a diamond wire Histo-Saw machine (Delaware Diamond Knives, Wilmington, DE, U.S.A.), and the thickness of each cross-sectional specimen was determined with an Inspectors' Dial Bench Gauge (L.S. Starrett, Athol, MA, U.S.A.). Frontal sections of the proximal tibia were cut at 8-µm thickness using a microtome (Leica RM 2155; Leica Inc., Nussloch, Germany). The sections were then transferred onto chro- malum-gelatin-coated slides and dried overnight in a press at 42°C. All sections were coverslipped.
with Eukitt (Calibrated Instruments, Hawthorne, NY, U.S.A.) for static histomorphometric analysis.

**Femoral length, wet weight, bone volume, and bone density**

The right femur was dissected free of soft tissue. The length of the bone was measured using a dial caliper. Then, the bone was placed in a volumetric flask filled with deionized water. The flask was placed in a desiccator under a vacuum for 2 h. After trapped air had diffused out of the bone, the wet weight of the bone was obtained using a Sartorius Balance (Northern Balance & Scale, Bloomington, MN, U.S.A.) with a thin wire to which the blotted bone was attached. The bone was weighed again after submersion in deionized water. The difference between the weight of the bone in air and that in water is BV. The wet weight and volume were used for the calculation of bone density.

**Bone densitometry**

After measurement of femoral wet weight and BV, BMD of the whole femur was determined by DXA using a Hologic QDR-2000 plus (Hologic Inc., Bedford, MA, U.S.A.). The instrument was adapted for an ultra-resolution mode with line spacing of 0.0254 cm, resolution of 0.0127 cm, and collimator of 0.9 cm diameter. The bones were placed in a petri dish. To simulate soft-tissue density surrounding the bones, tap water was poured around the bones to achieve a depth of 1 cm. The results were obtained as bone mineral content and bone area measured. BMD of this area was calculated as bone mineral content divided by bone area. Coefficients of variation of these measurements in our laboratory were less than 1.0%.

**Bone histomorphometry**

A digitizing morphometry system was used to measure bone histomorphometric parameters. The system consisted of an epifluorescence microscope (Olympus, BH-2; Olympus America Inc., Melville, NY, U.S.A.), a digitizing pad (Numonics 2206; Numonics Corp., Montomerville, PA, U.S.A.) coupled to an IBM computer, and a morphometry program (Osteo Metrics, Atlanta, GA, U.S.A.). The measured parameters for cancellous bone included total tissue volume (TV), BV, and bone surface (BS). These data were used to calculate percent cancellous BV (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), and trabecular separation (Tb.Sp) according to the standard nomenclature described by Parfitt et al. In this study, the region of cancellous bone measured was 1–4 mm distal to the lower margin of the growth plate in the proximal tibia, which consists of secondary spongiosus. The measured parameters for cortical bone were total tissue area (Tt.Ar), and cortical bone area (Ct.Ar). These data were used to calculate the marrow area (Ma.Ar).

**Serum calcium, phosphorus, and calcitropic hormones**

Upon death, serum was collected from all rats. The serum calcium and phosphorus levels were measured by an automated instrument (Dade Behring Model RXL, Bakersfield, CA, U.S.A.). The serum bioactive intact parathyroid hormone (PTH) level was measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit that was specific to rat PTH (ALPCO Diagnostics, Windham, NH, U.S.A.). The serum 1,25-dihydroxyvitamin D3 [1,25 (OH)2 D3] level was measured by a radioimmunoassay with calf thymus receptor using a kit manufactured by DiaSorin Inc. (Stillwater, MN, U.S.A.).

**Statistical analysis**

All data are presented as mean and standard deviation (SD). Multiple comparisons of data among the groups were performed by analysis of variance (ANOVA) with Fisher’s protected least significant difference (PLSD) test. The effect of supplementation of vitamin K and vitamin D was analyzed by two-way ANOVA. All statistical analyses were performed using the Stat View J-5.0 program (SAS Institute, Cary, NC, U.S.A.) on a Macintosh computer. A significance level of $p<0.05$ was used for all comparisons.
RESULTS

Body weight, and femoral length, wet weight, bone volume, bone density, and BMD

Table 1 shows body weight, and femoral length, wet weight, BV, bone density, and BMD. Initial body weight did not significantly differ among the nine groups. The maturation-related body weight gain was reduced in rats of the LC and LCK groups, but was increased in rats of the NCD group. Feeding a low calcium diet reduced the maturation-related gains in femoral length, wet weight, BV, bone density, and BMD. Vitamin K supplementation retarded the reductions in femoral bone density and BMD in rats fed a low calcium diet, and increased the maturation-related gains in femoral bone density and BMD in rats fed a normal calcium diet. On the other hand, vitamin D supplementation retarded the reductions in femoral length, wet weight, and BV in rats fed a low calcium diet, and increased the maturation-related gains in femoral length, wet weight, and BV in rats fed a normal calcium diet.

Bone histomorphometry of cancellous bone of proximal tibia

Fig. 1 shows histomorphometric parameters of cancellous bone of the proximal tibia and Table 2 shows the results of two-way ANOVA for histomorphometric analysis. Feeding a low calcium diet did not significantly influence the maturation-related cancellous BV/TV gain. However, whereas vitamin K supplementation increased this gain in rats fed a low calcium diet, it did not significantly influence the gain in rats fed a normal calcium diet. On the other hand, vitamin D supplementation reduced the maturation-related cancellous BV/TV gain in rats fed a low calcium diet, but increased it in rats fed a normal calcium diet.

Bone histomorphometry of cortical bone of tibial shaft

Fig. 2 shows the histomorphometric parameters

<p>| Table 1. Body Weight, and Femoral Bone Length, Wet Weight, Bone Volume, Bone Density, and BMD |
|-----------------------------------------------|---------|---------|---------|---------|---------|---------|---------|---------|</p>
<table>
<thead>
<tr>
<th>Group</th>
<th>Initial body weight (g)</th>
<th>Final weight (g)</th>
<th>Femoral length (mm)</th>
<th>Femoral wet weight (g)</th>
<th>Femoral bone volume (ml)</th>
<th>Femoral bone density (g/ml)</th>
<th>Femoral BMD (g/cm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLC (n=10)</td>
<td>115 ± 5</td>
<td>22.02 ± 0.39</td>
<td>0.276 ± 0.018</td>
<td>0.220 ± 0.013</td>
<td>1.255 ± 0.009</td>
<td>0.111 ± 0.007</td>
<td></td>
</tr>
<tr>
<td>NC (n=10)</td>
<td>110 ± 7</td>
<td>261 ± 13</td>
<td>34.22 ± 0.36*</td>
<td>0.764 ± 0.014*</td>
<td>0.487 ± 0.009*</td>
<td>1.571 ± 0.019*</td>
<td>0.221 ± 0.005*</td>
</tr>
<tr>
<td>NCK (n=10)</td>
<td>111 ± 7</td>
<td>269 ± 22</td>
<td>34.30 ± 0.38*</td>
<td>0.754 ± 0.025*</td>
<td>0.475 ± 0.016*</td>
<td>1.586 ± 0.017*</td>
<td>0.228 ± 0.003*</td>
</tr>
<tr>
<td>NCD (n=10)</td>
<td>119 ± 7</td>
<td>280 ± 19</td>
<td>34.70 ± 0.55*</td>
<td>0.807 ± 0.045*</td>
<td>0.511 ± 0.029*</td>
<td>1.581 ± 0.022*</td>
<td>0.226 ± 0.007*</td>
</tr>
<tr>
<td>NCKD (n=10)</td>
<td>111 ± 8</td>
<td>273 ± 20</td>
<td>34.42 ± 0.67*</td>
<td>0.817 ± 0.034*</td>
<td>0.508 ± 0.022*</td>
<td>1.607 ± 0.019*</td>
<td>0.227 ± 0.005*</td>
</tr>
</tbody>
</table>

Two-way ANOVA

K | ns | ns | ns | ns | ns | p<0.01 | p<0.05 |
D | ns | p<0.001 | p<0.001 | ns | ns |

LC (n=10) | 114 ± 5 | 242 ± 16 | 32.80 ± 0.35* | 0.514 ± 0.016* | 0.411 ± 0.009* | 1.249 ± 0.013* | 0.124 ± 0.006* |
LCK (n=10) | 117 ± 3 | 243 ± 13 | 33.20 ± 0.54* | 0.520 ± 0.015* | 0.408 ± 0.012* | 1.276 ± 0.009* | 0.130 ± 0.005* |
LCD (n=10) | 119 ± 7 | 248 ± 18 | 33.67 ± 0.47* | 0.538 ± 0.016* | 0.432 ± 0.015* | 1.245 ± 0.015* | 0.124 ± 0.004* |
LCKD (n=10) | 116 ± 6 | 250 ± 10 | 33.84 ± 0.47* | 0.547 ± 0.018* | 0.434 ± 0.015* | 1.262 ± 0.016* | 0.128 ± 0.004* |

Two-way ANOVA

K | ns | ns | ns | ns | ns | p<0.001 | p<0.001 |
D | ns | p<0.001 | p<0.001 | ns | ns |

Data are expressed as mean±SD. ANOVA with Fisher’s PLSD test was used to compare the data among the groups.

*Significantly different from BLC, †Significantly different from NC, ‡Significantly different from NCK, §Significantly different from NCD, ‡‡Significantly different from LC, ‡§Significantly different from LCK, ‡§§Significantly different from LCD.
of cortical bone of the tibial shaft and Table 2 shows the results of two-way ANOVA for histomorphometric analysis. Feeding a low calcium diet reduced the maturation-related Ct Ar gain as a result of decreased periosteal bone gain and enlarged the marrow cavity. Vitamin K supplementation retarded this reduction in rats fed a low calcium diet, and increased the maturation-related Ct Ar gain by reducing the marrow cavity in rats fed a normal calcium diet. On the other hand, vitamin D supplementation prevented the reduction in periosteal bone gain and enhanced the enlargement of the marrow cavity with no significant effect on the reduction in the maturation-related Ct Ar gain in rats fed a low calcium diet, and increased the maturation-related Ct Ar gain with increased periosteal bone gain in rats fed a normal calcium diet. An additive effect of vitamin K and vitamin D on the maturation-related Ct Ar gain was found in rats fed a normal calcium diet.
Fig. 2. Histomorphometric analysis of cortical bone. Data are expressed as mean±SD. ANOVA with Fisher’s PLSD test was used to compare the data among the groups. *: significantly different from BLC; a: significantly different from NC; b: significantly different from NCK; c: significantly different from NCD; d: significantly different from LC; e: significantly different from LCK. Two-way ANOVA showed the significant effect of vitamin K on Ct Ar and Ma Ar and the significant effect of vitamin D on Tt Ar and Ct Ar in rats fed a normal calcium diet, and the significant effect of vitamin K on Ct Ar and the significant effect of vitamin D on Tt Ar and Ma Ar in rats fed a low calcium diet (Table 2).

Table 2. Results of Two-Way ANOVA for Histomorphometric Analysis

<table>
<thead>
<tr>
<th></th>
<th>Cancellous bone</th>
<th>Cortical bone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BV/TV Tb N Tb Th Tb Sp</td>
<td>Tt Ar Ct Ar Ma Ar</td>
</tr>
<tr>
<td>Normal calcium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>n s</td>
<td>n s</td>
</tr>
<tr>
<td>D</td>
<td>p&lt;0.05</td>
<td>n s</td>
</tr>
<tr>
<td>Low calcium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>K</td>
<td>p&lt;0.01</td>
<td>n s</td>
</tr>
<tr>
<td>D</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

Serum calcium, phosphorus, and calcitropic hormones

Table 3 shows the levels of serum calcium, phosphorus, and calcitropic hormones. Feeding a low calcium diet induced hypocalcemia and increased the serum PTH level, thereby increasing the serum 1,25 (OH)₂ D₃ level as compared with feeding a normal calcium diet. Vitamin K supplementation did not prevent this hypocalcemia, but did retard this abnormal elevation of the serum PTH level with no significant influence on the serum 1,25 (OH)₂ D₃ level in rats fed a low calcium diet, and increased the serum 1,25 (OH)₂ D₃ level in rats fed a normal calcium diet. On the other hand, vitamin D supplementation prevented the abnormal elevation of the serum PTH level and hypocalcemia, and increased the serum 1,25 (OH)₂ D₃ levels in rats fed a low calcium diet, and increased the serum 1,25 (OH)₂ D₃ levels without alterations in the serum calcium and PTH levels in rats fed a normal calcium diet. An additive effect of vitamin K and vitamin D on intestinal calcium absorption was found only in rats fed a normal calcium diet, but no additive renal effect was found in rats fed either a normal or low calcium diet.

DISCUSSION

Feeding a low calcium diet induced hypocal
Table 3. Serum Calcium, Phosphorus, Creatinine, and Calcitropic Hormones

<table>
<thead>
<tr>
<th>Group</th>
<th>Serum calcium (mg/dl)</th>
<th>Serum phosphorus (mg/dl)</th>
<th>Serum PTH (pg/ml)</th>
<th>Serum 1,25-dihydroxyvitamin D (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLC (n=10)</td>
<td>10.10 ± 0.37</td>
<td>10.81 ± 0.32</td>
<td>156 ± 39</td>
<td>206 ± 46</td>
</tr>
<tr>
<td>NC (n=10)</td>
<td>10.26 ± 0.26</td>
<td>6.56 ± 1.11*</td>
<td>128 ± 83</td>
<td>58 ± 14*</td>
</tr>
<tr>
<td>NCK (n=10)</td>
<td>9.95 ± 0.25</td>
<td>6.41 ± 0.71*</td>
<td>149 ± 37</td>
<td>73 ± 14*</td>
</tr>
<tr>
<td>NCD (n=10)</td>
<td>9.97 ± 0.26</td>
<td>6.37 ± 0.56*</td>
<td>97 ± 32</td>
<td>82 ± 16*</td>
</tr>
<tr>
<td>NCKD (n=10)</td>
<td>10.05 ± 0.31</td>
<td>6.90 ± 0.75*</td>
<td>150 ± 67</td>
<td>98 ± 11*</td>
</tr>
</tbody>
</table>

Two-way ANOVA

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>D</td>
<td>n.s</td>
<td>n.s</td>
<td>n.s</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

LC (n=10) | 7.82 ± 0.59* | 7.21 ± 1.00* | 1087 ± 216* | 476 ± 49* |
LCK (n=10) | 8.04 ± 0.98* | 7.26 ± 0.77* | 651 ± 133* | 450 ± 75* |
LCD (n=10) | 9.88 ± 0.17* | 7.56 ± 0.41* | 186 ± 69* | 548 ± 11* |
LCKD (n=10) | 10.13 ± 0.25* | 10.21 ± 2.68* | 158 ± 52* | 592 ± 64* |

Two-way ANOVA

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>n.s</td>
<td>p&lt;0.01</td>
<td>p&lt;0.001</td>
<td>n.s</td>
</tr>
<tr>
<td>D</td>
<td>p&lt;0.001</td>
<td>p&lt;0.01</td>
<td>p&lt;0.001</td>
<td>p&lt;0.001</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. ANOVA with Fisher's LSD test was used to compare the data among the groups. The effects of vitamin K and vitamin D supplementation were analyzed by two-way ANOVA.

* Significantly different from BLC, †Significantly different from NC, ‡Significantly different from NCK, §Significantly different from NCD, ¶Significantly different from LC, ‰Significantly different from LCK, ††Significantly different from LCD.

cemia and increased the serum PTH and 1,25(D_{3}) levels. Feeding a low calcium diet also reduced the maturation-related gains in femoral length, wet weight, BV, bone density, and BMD, reduced the maturation-related cortical bone gain as a result of decreased periosteal bone gain and an enlarged marrow cavity, but did not significantly influence the maturation-related cancellous bone gain. Cancellous bone mass was little impacted. These results support the importance of adequate calcium intake to achieve normal skeletal growth, and suggest that cortical bone seems to be more responsive to calcium intake than cancellous bone. The absence of any significant alteration in cancellous bone mass in rats fed a low calcium diet may have resulted from redistribution of calcium from cortical bone where the development of osteopenia was evident.

Vitamin K supplementation retarded the reductions in femoral bone density and BMD in rats fed a low calcium diet, and increased the maturation-related gains in femoral bone density and BMD in rats fed a normal calcium diet. These results suggest that the effect of vitamin K on bone mass is characterized by improvement or increase in bone density rather than bone size in rats fed a normal or low calcium diet. Vitamin K supplementation also increased the maturation-related cancellous bone gain and retarded the reduction in the maturation-related Ct Ar gain in rats fed a low calcium diet, and increased the maturation-related Ct Ar gain by reducing the marrow cavity but did not significantly influence the maturation-related cancellous bone gain in rats fed a normal calcium diet. The retardation of the reduction in Ct Ar gain by vitamin K supplementation in rats fed a low calcium diet might have resulted from the combined non-significant retardation of the reduction in Tt Ar and the enlargement of the marrow cavity in...
Vitamin K, Vitamin D, and Bone Mass

321

terms of increased periosteal bone formation and decreased endocortical bone resorption, as compared with the state of calcium deficiency. These results suggest that vitamin K has an effect on cortical bone thickness in rats fed a normal or low calcium diet, and the potential to increase cancellous bone mass in rats fed a low calcium diet.

Vitamin K<sub>2</sub> is a cofactor of γ-carboxylase, which converts the glutamic acid (Glu) residue into γ-carboxyglutamic acid (Gla) residue in osteocalcin molecules, and is essential for the γ-carboxylation of bone Gla-containing protein of bone matrix (osteocalcin).<sup>14,15</sup> Available evidence suggests that vitamin K<sub>2</sub> also enhances osteocalcin accumulation in the extracellular matrix of osteoblasts in vitro.<sup>16</sup> Osteocalcin-knockout mice develop hyperostosis, suggesting that the Gla-containing osteocalcin promotes normal bone mineralization. The precise role of osteocalcin in bone mineralization remains obscure, but it probably regulates the growth of hydroxyapatite crystals. Vitamin K<sub>2</sub> has also been reported to inhibit the expression of the osteoclast differentiation factor (ODF)/RANK ligand, tartrate-resistant acid phosphatase activity, and mononuclear cell formation.<sup>17</sup> In addition, in unfraccionated bone cells and isolated osteoclasts on dentine slices, vitamin K<sub>2</sub> has been demonstrated to induce osteoclast apoptosis, suggesting that the inhibitory effect of vitamin K<sub>2</sub> on osteoclastic bone resorption may be exerted via the targeting of osteoclasts for apoptosis.<sup>18</sup> It has also been suggested that the inhibitory effect of vitamin K<sub>2</sub> on bone resorption may be independent on the γ-carboxylation system.<sup>19</sup> This line of evidence is substantiated by the anti-resorptive effect of vitamin K<sub>2</sub> on bone in vitro.

Over the past few years, several studies have demonstrated the effect of vitamin K<sub>2</sub> treatment on bone metabolism in animals with osteopenia in vivo, namely, an anabolic effect in sciatic neurectomized or prednisolone-treated rats<sup>20,21</sup> and an anti-resorptive effect in ovariectomized or orchidectomized rats.<sup>22</sup> Thus, vitamin K has the potential to regulate bone metabolism in vivo. The effects of vitamin K supplementation on renal and intestinal calcium retention have also been reported; vitamin K supplementation corrected hypercalciuria in vitamin K-deficient rats,<sup>7</sup> while vitamin K<sub>2</sub> supplementation improved renal calcium reabsorption in severe calcium/magnesium-deficient rats and increased intestinal calcium absorption in ovariectomized rats.<sup>23</sup> Thus, vitamin K has the potential to improve the calcium balance, and in particular, seems to have a renal effect under the condition of calcium deficiency. We speculate in this study that the positive effect of vitamin K supplementation on cancellous and cortical bone in rats fed a normal or low calcium diet might result from the improved or promoted balance of calcium excretion and absorption and bone resorption and formation.

Vitamin D supplementation retarded the reductions in femoral length, wet weight, and BV in rats fed a low calcium diet, and increased the maturation-related gains in femoral wet weight and BV in rats fed a normal calcium diet. These results suggest that the effect of vitamin D on bone mass is characterized by improvement or increase in bone size rather than bone density in rats fed a normal or low calcium diet. Surprisingly, very few studies have examined the effect of vitamin D supplementation on cortical and cancellous bone in rats fed a normal or low calcium diet. Vitamin D supplementation reduced the maturation-related cancellous bone gain, prevented the reduction in periosteal bone gain, and enhanced the enlargement of the marrow cavity with no significant effect on the reduction in maturation-related cortical bone gain in rats fed a low calcium diet. This result suggested that vitamin D supplementation prevented the reduction in the maturation-related periosteal bone gain by the accumulation of calcium from cancellous and endocortical bone under calcium deficiency. Vitamin D supplementation increased the maturation-related cancellous and cortical bone gains with increased periosteal bone gain in rats fed a normal calcium diet. The results of this study suggest that vitamin D has a strong effect on bone growth, in particular radial and longitudinal growth of the long bone in rats fed a normal or low calcium diet, and that adequate calcium supplementation may be needed to stimulate cortical bone growth and increase cancellous bone mass.
An animal study has shown that active vitamin D, alfacalcidol, has the potential to stimulate intestinal calcium absorption, increase urinary calcium excretion and serum calcium level, and suppress PTH secretion in the treatment of osteoporosis. Available evidence also suggests that vitamin D supplementation increases intestinal calcium absorption efficiency in postmenopausal women, and that this effect is regulated by 1,25 (OH)2 D3. Thus, it is well known that vitamin D stimulates intestinal calcium absorption via the increased serum 1,25 (OH)2 D3 level. With regard to the effect of vitamin D on bone metabolism, vitamin D supplementation has been shown to reduce bone loss in postmenopausal women by a direct effect on skeletal metabolism and an increase in intestinal calcium absorption. Alfacalcidol suppresses osteoclastic bone resorption and maintains or even stimulates bone formation at both cancellous and cortical bone sites in ovariectomized rats. It has been suggested that native vitamin D as well as alfacalcidol exerts bone-protective effects independently of its effects on calcium absorption. However, in this respect native vitamin D is inferior to alfacalcidol; the skeletal actions of alfacalcidol take place, at least in part, independently of the suppression of PTH secretion. Thus, native or active vitamin D has the potential to regulate bone metabolism. In this study, vitamin D supplementation might have increased periosteal bone formation or prevented its reduction in rats fed a normal or low calcium diet, and increased cancellous bone mass in rats fed a normal calcium diet by increasing intestinal calcium absorption and improving or promoting the balance of bone resorption and formation.

Available evidence suggests that the effect of vitamin K2 on mineralization by human periosteal osteoblasts is enhanced in the presence of 1,25 (OH)2 D3 in vitro. The effect of vitamin K2 on BMD in ovariectomized rats is affected by the plasma 25 (OH) D3 level in vivo, and is significant only when rats are fed a diet containing vitamin D3. Based on this line of evidence, combined treatment with vitamin D and vitamin K for osteoporosis is surmised to be more effective than either treatment alone. In this study, however, an additive effect was found only on cortical bone in rats fed a normal calcium diet probably via additively increased intestinal calcium absorption. Matsunaga et al. and Hirano and Ishii demonstrated the additive effect of vitamin K and vitamin D on bone mass in adult ovariectomized rats or young rats fed a normal calcium diet. Iwamoto et al. also demonstrated the efficacy of such combined treatment with vitamin K and vitamin D in postmenopausal women with osteoporosis under calcium supplementation. Thus, calcium supplementation may be needed to achieve the additive effects of vitamin K and vitamin D supplementation on bone.

Even though the effect of vitamin K and vitamin D supplementation was additive to the effect of vitamin D supplementation, this additive effect was quite minimal. This tendency was more apparent in cancellous bone than in cortical bone. According to the overall data of this study, the effects of vitamin K supplementation were generally modest, whereas the effects of vitamin D supplementation were generally more striking. In particular, vitamin D supplementation under the condition of normal calcium intake appeared to be helpful to stimulate the maturation-related bone gain. These differential efficacies of vitamin K and vitamin D may have been heightened by the lack of the minimal advantage of additional vitamin K supplementation over vitamin D supplementation alone.

This study featured two limitations. First, the effect of vitamin K and/or vitamin D on mechanical strength of cortical bone might be of interest, because the effect of these vitamins on cortical bone was quite different and because their additive effect was found only in cortical bone. Second, because an additive effect of vitamin K and vitamin D was found only in rats fed a normal calcium diet, the effect of vitamin K and/or vitamin D on cortical and cancellous bones in rats fed a high calcium diet may be of interest. Feeding a high calcium diet might show more significant advantages for vitamin K and/or vitamin D. Further studies are needed to test the bone mechanical strength and to examine the efficacy of vitamin K and/or vitamin D for bone growth under the condition of high calcium intake.

In conclusion, vitamin K supplementation in-
creased the maturation-related cancellous bone gain and retarded the reduction in maturation-
related cortical bone gain in rats fed a low calcium diet, and increased the maturation-
related cortical bone gain in rats fed a normal calcium diet. Vitamin D supplementation reduced the maturation-related cancellous bone gain, prevented the reduction in periosteal bone gain, and enhanced the enlargement of the marrow cavity, with no significant effect on the reduction in the maturation-related cortical bone gain in rats fed a low calcium diet, and increased the maturation-related cancellous and cortical bone gains with increased periosteal bone gain in rats fed a normal calcium diet. An additive effect of vitamin K and vitamin D on the maturation-related cortical bone gain was found in rats fed a normal calcium diet. This study has presented the differential effects of vitamin K and vitamin D supplementation on cancellous and cortical bone mass in young rats fed a normal or low calcium diet, as well as the additive effect of these vitamins on cortical bone under calcium sufficient condition.

REFERENCES

7. Robert D, Jorgetti V, Lacour B, Leclerq M, Cournot-
22. Akiyama Y, Hara K, Kobayashi M, Tomiyama T, Nakamura T. Inhibitory effect of vitamin K2 (mena-tetrenone) on bone resorption in ovariectomized rats: A histomophometric and dual energy X-ray absorp-

