Retraction

The following article has been retracted by the Editorial Board of Journal of Nutritional Science and Vitaminology, because some parts of their contents were published in other journals.

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Vitamin K2 Improves Femoral Bone Strength without Altering Bone Mineral Density in Gastrectomized Rats

Jun IWAMOTO1, Yoshihiro SATO2 and Hideo MATSUMOTO1

1 Institute for Integrated Sports Medicine, Keio University School of Medicine, Tokyo 160–8582, Japan
2 Department of Neurology, Mitate Hospital, Fukuoka 826–0041, Japan

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Summary Gastrectomy (GX) induces osteopenia in rats. The present study examined the skeletal effects of vitamin K2 in GX rats. Thirty male Sprague-Dawley rats (12 wk old) were randomized by the stratified weight method into the following three groups of 10 animals each: sham operation (control) group; GX group; and GX+oral vitamin K2 (menatetreone, 30 mg/kg, 5 d/wk) group. Treatment was initiated at 1 wk after surgery. After 6 wk of treatment, the bone mineral content (BMC), bone mineral density (BMD), and mechanical strength of the femoral diaphysis and distal metaphysis were determined by peripheral quantitative computed tomography and mechanical strength tests, respectively. GX induced decreases in the BMC, BMD, and ultimate force of the femoral diaphysis and distal metaphysis. Vitamin K2 did not significantly influence the BMC or BMD of the femoral diaphysis or distal metaphysis in GX rats, but attenuated the decrease in the ultimate force and increased the stiffness of the femoral diaphysis. The present study showed that administration of vitamin K2 to GX rats improved the bone strength of the femoral diaphysis without altering the BMC or BMD, suggesting effects of vitamin K2 on the cortical bone quality.

Key Words gastrectomy, cortical bone, bone strength, bone mineral density, vitamin K

Gastric surgery is mostly needed for the treatment of gastric cancer. Gastrectomy (GX) in patients results in malnutrition and bone diseases, including not only osteoporosis but also osteomalacia or a mixed pattern of osteoporosis/osteomalacia with secondary hyperparathyroidism (1, 2). The risk of fractures is increased in GX patients (3–8). This risk is sustained even when patients are cured of their disease (9). Since GX induces impairment of calcium and vitamin D metabolism (5, 10), calcium and vitamin D supplementation may play an important role in maintaining bone health (7).

However, vitamin K insufficiency as well as vitamin D insufficiency and deficiency owing to malnutrition are speculated to be partly associated with the increased risk of fracture in GX patients, similar to the case for patients with neurological diseases (11–13). Vitamin K insufficiency has been suggested to increase the risk of hip fractures in elderly people (14–17). Meta-analyses have demonstrated the anti-fracture efficacy of vitamin K2, which is widely used for treatment of osteoporosis in Asia. In patients with an increased risk of fracture (18, 19). Thus, it is surmised that vitamin K2 therapy could be useful for maintaining bone health in GX patients.

An animal model of cortical and cancellous osteopenia can be created by performing GX in rats. Total GX and resection of the acid-producing part of the stomach (fundectomy) induce gastrinemia and malnutrition (20, 21), thereby initiating trabecular and cortical osteopenia in rats (22–26). The hypothesis of the present preclinical study was that vitamin K2 would improve the bone strength in GX rats. Thus, experiments were conducted to determine the skeletal effects of vitamin K2 in GX rats. The primary endpoint was the bone strength at sites rich in trabecular or cortical bone. The secondary endpoints included the bone weight, bone mineral content (BMC), and bone mineral density (BMD).

MATERIALS AND METHODS

Handling of animals. Thirty male Sprague-Dawley rats (11 wk old) were purchased from Charles River Japan (Kanagawa, Japan). The rats were housed in a local animal room with a temperature of 24°C, humidity of 50%, and 12-h/12-h light/dark cycle, and were allowed free access to water and a standard pellet diet containing 1.25% calcium and 0.9% phosphorus (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan). After 1 wk of adaptation to this environment, the 12-wk-old rats were sorted into strata by body weight and then randomized by the stratified weight method into the following three groups of 10 animals each: sham operation control (CON) group; GX group; and GX+vitamin K2 group. Total GX was performed under general anesthesia with 25–30 mg/kg of pentobarbital (Kyoritsu Seiyaku Co. Ltd., Tokyo, Japan) injected intraperitoneally together with 2–3% isoflurane (Mylan Inc., Tokyo, Japan) delivered via a Table Top Laboratory Animal Anesthesia System (V1 Type VetEquip Inc., Pleasanton, CA). A longitudinal incision was made on the abdomen to expose the stomach. The whole stomach was excised, and an anastomosis of the esophagus and duodenum was performed. Treatment was initiated at 1 wk after surgery. In the CON and GX groups, vehicle was administered by gavage five times a
week. In the GX+vitamin K₂ group, vitamin K₂ (menaquinone-4, menatetrenone; Eisai Co. Ltd., Tokyo, Japan) was suspended in a fatty acid preparation (Miglyol 812; Mitsuba Trading Co. Ltd., Tokyo, Japan) at a dosage of 30 mg/mL/kg and administered by gavage five times a week. Although the dosage may be considered to be very high, treatment with vitamin K₂ at 30 mg/kg five times a week has previously been reported to maintain bone health in rats without causing any adverse events (27, 28). The weight of the rats was monitored weekly and the duration of the treatment was 6 wk. The study was carried out at the laboratory of Hamri Co. Ltd. (Ibaraki, Japan), which has been approved by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Hamri Co. Ltd.

Preparation of specimens. At 6 wk after the start of treatment, the animals were euthanized by exsanguination under general anesthesia with 2–3% isoflurane using the above-described Table Top Laboratory Animal Anesthesia System. The bilateral femora were harvested from each animal. The left femur was used for measurements of the length with a dial caliper and the weight on an electronic balance (A&D Company, Tokyo, Japan), which has been approved by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Hamri Co. Ltd.

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| pQCT of the femur. The distal metaphysis and diaphysis of the femur were scanned by pQCT (XCT-Research SA+; Stratec Medizintechnik GmbH, Pforzheim, Germany) in 70% ethanol/saline. The bones were placed horizontally in a polypropylene tube and scanned at a voxel size of 0.12 mm. The scan line was adjusted using the scout view, and sites at 3 and 14 mm proximal to the distal growth plate were scanned. For analysis, a threshold of 395 mg/cm³ in contour mode 2 was used to separate the bone tissue from the marrow in the distal metaphysis. The total and trabecular BMC, BMD, and area were evaluated. A constant threshold of 690 mg/cm³ in contour mode 1 was used to separate the cortical bone from the trabecular bone in the diaphysis. The BMC, BMD, area, thickness, and periosteal and endocortical perimeters were evaluated. |

| Mechanical strength test of the femur. Each specimen was submerged for 1 h before testing to allow temperature equilibration. The mechanical strength of the femoral diaphysis was evaluated by a three-point bending test. A load was applied to the bone midway between two supports placed 18 mm apart. The femur was positioned so that the loading point was at the center of the femoral diaphysis, and bending occurred at the medial–lateral axis. Load-displacement curves were recorded at a crosshead speed of 5 mm/min using a material-testing machine (MZ-500S; Maruto Instrument Co. Ltd., Tokyo, Japan). The parameters analyzed were the stiffness, work to failure, and ultimate force. |

| Just after the three-point bending test of the femoral diaphysis, the distal metaphysis of the femur was isolated over a length of 10 mm from the joint surface of the femoral condyle. The mechanical strength of this segment was then measured by a compression test. A compressive load was applied by a rectangular parallelepiped crosshead (length, 2 cm; width, 2 cm; height, 1 cm) to the femoral distal metaphysis from the lateral aspect to the medial aspect. The specimens were positioned so that the loading point was at the center of the femoral lateral condyle. Load-displacement curves were recorded at a crosshead speed of 5 mm/min and a compression depth of 2.5 mm using a material-testing machine (MZ500D; Maruto Instrument Co.). The parameters analyzed were the stiffness, work to failure, and ultimate force. |

| Statistical analysis. Data were expressed as the mean±standard deviation (SD). Comparisons among the groups were performed by analysis of variance (ANOVA) using Fisher’s protected least significant difference (PLSD) test. All statistical analyses were carried out using the Stat View j-5.0 program on a Windows computer and significance was accepted at p<0.05. |

RESULTS

Body weight, femoral length, and femoral weight

As shown in Table 1, the initial body weights at surgery did not differ significantly among the three groups. The body weights at baseline (1 wk after surgery, but
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Before treatment) and after 6 wk of treatment were lower in the GX group than in the CON group. Vitamin K2 administration to GX rats did not significantly influence the body weight.

After 6 wk of treatment, the femoral lengths did not differ significantly between the CON and GX groups. However, the femoral length was lower in the GX vitamin K2 group than in the CON and GX groups. The femoral weight was lower in the GX group than in the CON group. Vitamin K2 administration to GX rats did not significantly influence the femoral weight.

**pQCT analysis of the femur**

Table 2 shows the results of the pQCT analysis of the femoral diaphysis after 6 wk of treatment. GX caused decreases in the total BMC, and total and trabecular BMD and increases in the trabecular BMC and area, but did not cause any significant change in the total area. The mean GX-induced decrease rates of the total BMC and BMD were 33.3% and 32.4%, respectively. The corresponding change rates of the trabecular BMC and BMD were +40.2% and −26.4%, respectively. Vitamin K2 administration to GX rats did not significantly improve the total or trabecular BMC and BMD or trabecular area, but decreased the total area.

**Mechanical strength of the femur**

Figure 1 shows the results of the mechanical strength tests of the femoral diaphysis and distal metaphysis after 6 wk of treatment. In the femoral diaphysis, GX induced a decrease in the ultimate force, but did not cause any significant change in the stiffness or work to failure. Vitamin K2 administration to GX rats attenuated the decrease in the ultimate force and increased the stiffness, but did not significantly change the work to failure.

In the femoral distal metaphysis, GX induced decreases in the stiffness, work to failure, and ultimate force. Vitamin K2 administration to GX rats did not significantly improve any of these parameters.

| Table 2. pQCT data for the femoral diaphysis. |
|-----------------------------------------------|
| **CON** (n=10)                                |
| **GX** (n=10)                                 |
| **GX+vitamin K2** (n=10)                      |
| BMC (mg/mm)                                   |
| 10.9±0.9                                      |
| 8.4±0.6a                                      |
| 8.3±0.9a                                      |
| BMD (mg/cm³)                                  |
| 1,372±14                                      |
| 1,328±17a                                     |
| 1,311±26a                                     |
| Area (mm²)                                    |
| 7.93±0.62                                     |
| 6.32±0.37a                                    |
| 6.35±0.58a                                    |
| Thickness (mm)                                |
| 0.81±0.04                                     |
| 0.61±0.05a                                    |
| 0.61±0.05a                                    |
| Periosteal perimeter (mm)                     |
| 12.4±0.5                                      |
| 12.3±0.4                                      |
| 12.4±0.5                                      |
| Endocortical perimeter (mm)                   |
| 7.28±0.39                                     |
| 8.48±0.56a                                    |
| 8.55±0.47a                                    |

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

a Significant difference vs. CON.

CON: control, GX: gastrectomy, pQCT: peripheral quantitative computed tomography, BMC: bone mineral content, BMD: bone mineral density.

| Table 3. pQCT data for the femoral distal metaphysis. |
|-------------------------------------------------------|
| **CON** (n=10)                                       |
| **GX** (n=10)                                        |
| **GX+vitamin K2** (n=10)                             |
| BMC (mg/mm)                                           |
| Total 13.2±1.1                                       |
| Trabecular 1.79±0.41                                 |
| 8.8±1.1a                                           |
| 2.51±0.42a                                         |
| 2.18±0.68a                                          |
| BMD (mg/cm³)                                         |
| Total 525±39                                        |
| Trabecular 212±28                                    |
| 355±38a                                             |
| 156±36a                                            |
| 361±36a                                            |
| 140±31a                                             |
| Area (mm²)                                           |
| Total 25.1±1.8                                      |
| Trabecular 8.6±2.2                                   |
| 24.8±1.1                                           |
| 16.3±1.3a                                          |
| 23.1±1.7ab                                          |
| 15.4±1.6a                                           |

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

a Significant difference vs. CON, b significant difference vs. GX.

CON: control, GX: gastrectomy, pQCT: peripheral quantitative computed tomography, BMC: bone mineral content, BMD: bone mineral density.
DISCUSSION

The present study examined the skeletal effects of vitamin K2 in GX rats. The key findings were: 1) influences of GX on the BMC, BMD, and bone strength of the femoral diaphysis and distal metaphysis in rats; 2) lack of significant influences of vitamin K2 administration to GX rats on the BMC and BMD of the femoral diaphysis and distal metaphysis; and 3) effects of vitamin K2 administration to GX rats on the bone strength of the femoral diaphysis.

Gastric acid is thought to facilitate the intestinal absorption of ingested calcium by mobilizing calcium from insoluble complexes in the diet (22). After GX in humans, pigs, and rats, serum gastrin decreases, calcium absorption is impaired, serum calcium and 25(OH)D decrease, and 1,25(OH)2 D increases (5, 10, 29–31). GX in rats induces osteopenia (reduced BMC and BMD) (21, 25, 26, 32–34). However, trabecular bone loss appears to be greater than cortical bone loss (25, 35), and the inner diameters of the tubular bones (femur and tibia) are greatly increased (36). In the present study, GX decreased the BMC and BMD of the femoral diaphysis and distal metaphysis, with greater decreases in the femoral distal metaphysis than in the femoral diaphysis. The endocortical perimeters of the femoral diaphysis and the trabecular area of the femoral distal metaphysis were increased. These findings are consistent with those in previous studies (21, 25, 26, 32–35). The increase in the trabecular BMC of the femoral distal metaphysis may be attributable to the increase in the trabecular area.

A preclinical study showed that bisphosphonate incadronate prevented GX-induced osteopenia and osteomalacia in rats (29). Theoretically, however, bisphosphonates may be a contraindication for osteomalacia (37), and not only calcium and vitamin D, but also drugs that increase bone mineralization, may be suitable therapies for bone disorders after GX. Vitamin K is known to be a cofactor of γ-carboxylase, which converts glutamic acid (Glu) residues in osteocalcin molecules to γ-carboxyglutamic acid (Gla) residues and is therefore essential for γ-carboxylation of osteocalcin (38–40). Without this modification, osteocalcin lacks structural integrity and the ability to bind to the hydroxyapatite mineral. Thus, vitamin K2 is likely to play a role in mineralization of bone and can be a good candidate for the treatment of bone disorders after GX.

In the present study, however, vitamin K2 improved the ultimate force and stiffness of the femoral diaphysis without altering the BMC or BMD in GX rats, suggesting that it had effects on the cortical bone quality.
Several preclinical studies have suggested effects of vitamin K2 on bone strength or bone quality (matrix volume, osteocyte density, and collagen cross-links), but not on bone mass, in rats (41–47). Clinically, Tanaka et al. (48) reported that the osteocalcin/deoxypyridinoline and osteopontin/calcium ratios of cortical bone were lower in patients with femoral neck fractures than in those with osteoarthritis of the hip. Iwamoto et al. (18) reported the anti-fracture efficacy of vitamin K2 against fractures of the hip, a skeletal site rich in cortical bone, in patients with neurological diseases. These reports support a significant influence of osteocalcin and a beneficial effect of vitamin K2 on cortical bone quality and/or strength.

Vitamin K2 decreased the femoral length and femoral distal metaphyseal total area in GX rats. These results suggest the retardation of bone growth, which could be a limitation of high dose vitamin K2 administration. Further studies are needed to determine whether there was a toxicity of high dose vitamin K2 on the skeleton.

The body weight markedly decreased after GX in rats, suggesting an induction of malnutrition by GX. It is well known that GX causes impairment of calcium absorption and vitamin D insufficiency or deficiency. However, vitamin K insufficiency could coexist in GX rats owing to malnutrition. Nevertheless, vitamin K nutritional status after GX has rarely been studied. Serum levels of vitamin K1, vitamin K2, and undercarboxylated osteocalcin, which increases according to vitamin K insufficiency because of impairment of γ-carboxylation of osteocalcin, appear to be good indices of vitamin K nutritional status. Thus, the assessment of these parameters could reveal the existence of vitamin K insufficiency after GX and the beneficial effect of vitamin K2 on the skeleton in GX rats. Further studies are needed to clarify these issues.

The present study had several limitations. First, we did not evaluate serum or urinary calcium or phosphorus, serum 25(OH)D, 1,25(OH)2D, parathyroid hormone (PTH), or bone turnover markers. The effects of vitamin K2 on these biochemical parameters may be of importance for translating the results of our preclinical study into clinical practice. Second, we used young male rats because previous studies have reported the influence of GX on bone parameters in young male rats. Thus, the results of the present study may be relevant to GX in men. GX is associated with a significant risk of hip fractures in men (4). However, ovarioctomized aged rats may be more interesting to study from the point of view of the increased burden of osteoporosis among elderly women after GX, although it may not be easy for aged rats to survive after GX. Thus, further studies are needed to establish the skeletal effects of vitamin K2 in a rat model of elderly women with osteoporosis after GX.

In conclusion, the present study showed that GX induced decreases in the BMC, BMD, and ultimate force (bone strength) of the femoral diaphysis and distal metaphysis, and that administration of vitamin K2 to GX rats improved the ultimate force and stiffness of the femoral diaphysis without altering the BMC or BMD.

These findings suggest effects of vitamin K2 on the quality and strength of cortical bone. It will be of interest to determine whether vitamin K2 can prevent fractures in GX patients by conducting clinical trials.

Disclosures

All the authors state that they have no conflicts of interest.

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