Menatetrenone Ameliorates Reduction in Bone Mineral Density and Bone Strength in Sciatic Neurectomized Rats

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Summary Menatetrenone (MK-4) is a vitamin K2 homologue that has been used as a therapeutic agent for osteoporosis in Japan. Rat models of immobilization induced by sciatic neurectomy are characterized by transiently increased bone resorption and sustained reduction in bone formation. Using such a rat model, we investigated the efficacy of MK-4 on bone loss. Male Sprague-Dawley rats were subjected to unilateral sciatic neurectomy and administered MK-4 for 28 d beginning day 21 after operation. The effect of MK-4 on the immobilized bone was assessed by measuring the bone mineral density of the femur, breaking force of the femoral diaphysis, and bone histomorphometry in tibial diaphysis. The BMD on both the femoral distal metaphysis and diaphysis was reduced by sciatic neurectomy. The administration of MK-4 ameliorated this reduction in a dose-dependent manner. The administration of 30mg/kg MK-4 ameliorated the reduction in bone strength. An improvement in bone formation was observed following the administration of MK-4. These results suggest that MK-4 has a therapeutic potential for immobilization-induced osteopenia.

Key Words menatetrenone, bone mineral density, bone strength, cortical bone, sciatic neurectomy

Vitamin K is known to promote the post-translational modification of vitamin K-dependent proteins such as osteocalcin, matrix Gla protein, and protein-S. Osteocalcin is a protein that contains γ-carboxyglutamic acid, is found in the bone matrix, and is synthesized only by osteoblasts. Osteocalcin contains three residues of γ-carboxyglutamic acid (Gla) at positions 17, 21, and 24 of the amino acid sequence, which are responsible for its high affinity for hydroxyapatite. Plasma osteocalcin concentration is used clinically as a biochemical marker of bone formation.

Vitamin K exists in two forms, vitamin K1 and vitamin K2, in nature. Vitamin K1 is contained in seaweed and green plants. Vitamin K2 is contained in cheese, egg yolk, and fermented soybeans (natto), and is also synthesized by bacteria in the bowel. In more recent studies, dietary vitamin K intake was found to be associated with a high incidence of femoral neck and vertebral fractures (1, 2). Soybeans fermented (natto) by Bacillus natto, a traditional Japanese food, contain 100 times more vitamin K2 (mainly menaquinone-7) than cheese (3). Two reports have suggested that natto consumption is associated with the frequency of hip fracture and bone mineral density in Japanese women (4, 5).

Menatetrenone (MK-4) is a vitamin K2 homologue that has been used as a therapeutic agent for osteoporosis in Japan. Recent clinical studies have suggested that MK-4 effectively prevents the occurrence of new fractures in patients with postmenopausal osteoporosis (6–8) and ameliorates osteoporosis associated with Parkinson’s disease (9). The effects of MK-4 on bone metabolism were demonstrated in a model of postmenopausal osteoporosis (10–12). It is generally accepted that bone resorption is increased in ovariectomized rats. Various studies have suggested that MK-4 prevents, to some extent, the bone loss induced by ovariectomy by inhibiting bone resorption (10, 11). We reported that MK-4 protected against the loss of trabecular bone volume and its structure in rats rendered osteoporotic by sciatic neurectomy, through the process of maintaining bone formation (13). However, these studies were intended to demonstrate the protective effect of MK-4 and analyze cancellous bone sites. It has not yet been elucidated as to whether MK-4 ameliorates reduced BMD in cortical bone in vivo.

Unloading of bone by immobilization such as that due to long-term bed rest, paralysis, after spinal cord injury, and plaster cast fixation leads to systemic or local bone loss known clinically as disuse osteopenia.
Bone volume loss has been recognized as a one of the major health problems affecting elderly patients with bone fractures. A number of immobilization methods have been used to induce experimental disuse osteopenia. Models of single-leg immobilization include wrapping or casting the leg, tenotomy, neurotomy, neurectomy, and hemicordotomy. Tenotomy, neurotomy or neurectomy, and hemicordotomy cause a rapid decrease in bone mass in experimental animals (14–19). Unilateral sciatic neurectomy is a simple surgical procedure and has been used by many investigators with satisfactory results.

The purpose of this study was to evaluate the effect of MK-4 on established bone loss induced by immobilization, as a surrogate to estimate fracture risk, by measuring bone mineral density (BMD), bone mechanical properties, and bone histomorphometry.

MATERIALS AND METHODS

Experimental protocol. Forty-two male Sprague-Dawley rats (Nippon Bio Supply Center Co., Ltd., Tokyo, Japan) weighing approximately 440 g were acclimated to our vivarium conditions (12-h light/dark cycle at 23 ± 1°C) for 7 d. During the experimental period, the rats were allowed free access to water and standard powder feed (#92095, Harlan Teklad, Madison, WI, USA), which contained 0.7% (w/w) calcium and 0.6% (w/w) phosphorus. They were checked daily and their body weight and food intake were measured three times per week to monitor their general health. Six animals were sacrificed at the beginning of the experiment to obtain baseline data. The rest of the rats were divided into two groups and were subjected to unilateral sciatic neurectomy (n = 24) or sham operation (n = 12). Sciatic neurectomy was performed on the right hindlimb. Rats were anesthetized with an intraperitoneal injection of pentobarbital sodium, a dorsolateral incision was made on the right hip through which the sciatic nerve was exposed, a 0.5 cm segment was excised, and the muscle and skin were sutured to its case. The sham-operated control rats underwent an operation similar to that of the unilateral sciatic neurectomy group, except that the nerve was mobilized but not transected.

Three weeks after neurectomy, six neurectomized rats (SNx) and six sham-operated rats were sacrificed to obtain pre-administration data (day 21). The rest of the neurectomized rats were divided into three groups. SNx rats were administered either 10 mg/kg MK-4 (SNx + LK), 30 mg/kg MK-4 (SNx + HK), or the vehicle (SNx + vehicle). MK-4 (menatetrenone; Eizai Co. Ltd., Tokyo, Japan) was given as a dietary supplement 30 mg/100 g, 85 mg/100 g of the diet, respectively. The dosages of MK-4 were set based on the results of earlier studies (10, 12, 13). Sham-operated and immobilized rats were sacrificed on day 49.

The animals were treated humanely, in compliance with the regulations of Oita University of Nursing and Health Sciences.

Serum biochemistry. Blood samples were obtained from the abdominal aorta at the time of sacrifice. Serum Ca, Pi, and alkaline phosphatase activity were measured in an autoanalyzer (Hitachi 736, Hitachi Co. Ltd., Hitachi, Japan). Serum PTH levels were measured by immunoradiometry using a kit for rat PTH (Nichols Institute, San Juan Capistrano, CA, USA). Serum levels of 1,25(OH)2D3 were measured by radioreceptor assay using vitamin D receptors derived from calf thymocytes. The serum MK-4 concentration was measured by high-performance liquid chromatography according to the method of Langenberg and Tjaden (20) in samples from day 49.

Bone mineral densitometry (BMD). At the designed determination points, bone samples were taken and all the connective tissues were carefully removed. BMD was measured by directly applying radiation beams. The BMD of the right femur was determined by dual energy X-ray absorptiometry (DXA) using QDR-1500 (Hologic, Waltham, MA, USA). The ultrahigh resolution scan mode (rat mode, Version 4.59 software, Waltham) was used. The BMD of the distal one-fourth of the femur, including the epimetaphyseal region, and of the middle half of the femur representing the cortical diaphyseal region, was measured. The coefficient of variation of BMD was 0.95% in the rat mode using the step phantom for small animals.

Bone mechanical properties. The three-point bending test was performed on the femur using an Autograph (AG-2000E, Shimadzu, Kyoto, Japan) according to the instructions provided by the manufacturer. Using a 1.0 cm sample, the center of the femur was fractured by applying perpendicular pressure at a speed of 100 mm/min.

Bone histomorphometry. All rats were subcutaneously injected with calcine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) (8 mg/kg body weight) 7 and 3 d before sacrifice. Upon sacrifice, the right tibia was removed and frozen under liquid nitrogen. Upon fixation in 70% ethanol and embedding in methyl-methacrylate (Wako Pure Chemical Industries) without decalcification. Cross-sections of the tibial shaft proximal to the tibiofibular junction were cut at a thickness of 30 µm using a Leica saw microtome SP1600. Sections were used to clearly visualize calcine under fluorescent light microscopy. Two-dimensional parameters, obtained by histomorphometry of the cortical bone, were performed using semi-automated systems (Osteoplan II; Carl Zeiss, Thornwood, NY, USA). The endosteal bone surface (BS) and lengths of the single-labeled surface (sLS) and double-labeled surface (dLS) were traced at ×200. Labeling width was calculated as the average distance between the double labels, and the mineral apposition rate (MAR, µm/d) was calculated by dividing the labeling width by the number between the two calcine administrations. Bone formation rate per bone surface (BFR/BS, µm2/µm2/d) was the product of (sLS/2 + dLS) × MAR/BS. The nomenclature, symbols, and units used in this study are those recommended by the American Society for Bone Mineral Research (ASBMR) Nomenclature Committee (21).
Statistical analysis. All data are expressed as the mean value±standard deviation (SD), and the statistical analyses were performed by one-way analysis of variance (ANOVA). A p value less than 0.05 was considered to indicate statistical significance.

RESULTS

General conditions, body weights and food intake

All the rats remained healthy during the experimental period. On day 21, body weights were 503.3±15.3 g and 456.7±15.3 g in the sham-operated and SNx groups, respectively. No difference in final body weight, body weight gain, food intake, behavior, or appearance was observed among the groups (Table 1). The actual intake of menatretrenone was 10.9±0.3, 31.2±0.6 mg/kg body wt/d, respectively, as calculated by the average diet intake and menatetrenone content in the diet.

Serum biochemistry

Serum parameters on day 49 are shown in Table 2. There was no difference in the serum levels of Ca, Pi, or Alp among the main four groups (i.e., sham-operated, SNx+vehicle, SNx+LK and SNx+HK) at any determination point. In additional, the serum concentrations of PTH and 1,25(OH)2D3 were similar among the SNx groups (data not shown).

Bone mineral density as assessed by DXA

On day 21 after the operation, the BMD of the femoral distal metaphysis was significantly lower in the SNx rats as compared with that of the sham-operated rats. On day 49, the BMD in the femoral distal metaphysis was significantly higher as compared with the value of day 21 in the MK-4-administered groups, whereas BMD continued to fall in the SNx+vehicle group (Fig. 1a). On day 49, there was no difference in cortical bone area measured by DXA among the SNx groups (data not shown). Bone mineral content (BMC) was 0.240±0.011 g in the SNx+vehicle group, 0.239±0.006 g in

![Fig. 1a. Recovery of BMD in the femoral distal metaphysis after MK-4 administration. MK-4 was given from day 21 to 49. MK-4 administration and BMD measure-ment were as described in Materials and Methods. Data are the mean value±SD of six animals. Solid column: sham-operated group, open column: SNx+vehicle group, dot column: SNx+LK group, striped column: SNx+HK group. *p<0.05 compared with the sham-operated group. #p<0.05 compared with the SNx+vehicle group. +p<0.05 compared with the SNx group on day 21.

![Fig. 1b. The BMD of the femoral diaphysis at the end of the experimental period. MK-4 was given from day 21 to 49. MK-4 administration and BMD measurement were as described in Materials and Methods. Data are the mean value±SD of six animals. *p<0.05 compared with sham-operated group. #p<0.05 compared with SNx+vehicle group. *p<0.05 compared with SNx+vehicle group.]

Table 1. Body weights and food intake after the MK-4 administration period.

| Groups        | Final body weight (g) | Body weight gain (g/d) | Food intake (g/d) |
|---------------|-----------------------|------------------------|-------------------|
| Sham          | 559.3±12.0            | 1.47±0.47              | 18.5±0.4          |
| SNx+vehicle   | 492.3±47.0            | 1.30±0.22              | 18.0±0.6          |
| SNx+LK        | 494.0±25.2            | 1.30±0.35              | 17.9±0.4          |
| SNx+HK        | 491.1±26.5            | 1.29±0.51              | 18.0±0.2          |

The data of body weight gain [final body weight−day 21 body weight] was obtained for each rat. All values are expressed as the mean value±SD.

Table 2. Parameters in serum on day 49.

| Groups        | Ca(mg/dL)   | IP(mg/dL)   | Alp(IU)    | MK-4(ng/mL) |
|---------------|-------------|-------------|------------|-------------|
| Sham          | 10.3±1.7    | 8.9±3.1     | 426±129    | 2.5±1.8     |
| SNx+vehicle   | 11.0±0.4    | 9.7±1.3     | 565±80     | 3.4±2.4     |
| SNx+LK        | 11.7±1.3    | 12.6±2.3    | 433±70     | 379.8±1047††|
| SNx+HK        | 11.1±0.9    | 11.1±12.5   | 470±21     | 2444.0±623.0††|

Data are the mean value±SD of six animals. *p<0.05 compared with the sham-operated group. #p<0.05 compared with the SNx+vehicle group.
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Fig. 2 Effect of MK-4 on the strength of the femoral diaphysis. The breaking force measurement was as described in Materials and Methods. Data are the mean value±SD of six animals. * p<0.05 compared with the sham-operated group. # p<0.05 compared with the SNx+vehicle group.

Bone mechanical properties

As shown in Fig. 2, the breaking force in the SNx+vehicle group was lower as compared to that of the sham group. In the group administered 30 mg/kg of MK-4, the value of this parameter was nearly comparable to that of the sham-operated group.

Bone formation in tibial cortical bone

Table 3. Effect of MK-4 on bone formation parameters in tibial diaphysis after 28 d of treatment.

|       | MS/BSI(%) | MAR(μm/d) | BFR/BSI(μm³/μm²/d) |
|-------|-----------|------------|---------------------|
| Sham  | 50.38±7.93| 2.08±0.53  | 1.05±0.03           |
| SNx+vehicle | 10.40±1.52  | 0.10±0.01* | 0.01±0.00*          |
| SNx+LK | 56.47±3.99* | 1.57±0.13* | 0.09±0.06*          |
| SNx+HK | 67.25±1.25** | 2.17±0.26** | 1.44±0.06**         |

Table 3. Effect of MK-4 on bone formation parameters in tibial diaphysis after 28 d of treatment.

MK-4 was given from day 21 to day 49. MK-4 administration and bone histomorphometry were as described in Materials and Methods.

DISCUSSION

The prevention of bone fracture is a major health problem since fractures seriously affect a patient's quality of life. In the present study, we examined the effect of MK-4 on reduced bone mineral density induced by sciatic neurectomy. Immobilization-induced osteopenia is characterized by both a sustained decrease in bone formation and transient increase in bone resorption, resulting in rapid bone loss (14). In previous reports, bone loss was observed within the first 10 d of immobilization (14, 15). Loss of bone mineral density induced by sciatic neurectomy occurred within 3 wk after the operation. This observation was suitable for expeditious evaluation of the efficacy of MK-4, an anabolic and antiresorption agent.

After 28 d of treatment with 30 mg/kg MK-4, BMDs in the femoral distal metaphysis and diaphysis were ameliorated in immobilized rats. The supplementation of 30 mg/kg MK-4 also significantly ameliorated the decrease in bone strength at the femoral diaphysis. We previously reported that MK-4 protected against the loss of trabecular bone structure as well as bone volume in rats rendered osteoporotic by sciatic neurectomy (13). Both the increase in BMD and strength of the femoral diaphysis indicate that MK-4 may play an effective role in not only cancellous bone, but also in cortical bone.

In previous reports (22, 23), there were no differences in the levels of serum calcium, phosphorus, alkaline phosphatase, or PTH in sciatic neurectomized rats as compared to sham-operated rats. However, these reports did not assess the 1,25(OH)₂D₃ concentration, which may not change in sciatic neurectomized rats. Since no significant changes were observed in the serum concentrations of the calcium and calcitropic hormones among the SNx groups, the effect of MK-4 on bone metabolism seems to be related to γ-carboxylation on the bone cell and not due to the enhancement of providing mineral to bone mediated through augmented intestinal absorption of calcium and phosphate with alteration of the calcitropic hormones.

MK-4 inhibits bone resorption in vitro (24–27) and in vivo (10–13) and enhances osteoblast-induced mineralization in vitro (28, 29). We have previously reported the protective effect of MK-4 on trabecular bone loss induced by sciatic neurectomy (13). This report suggests that the preventive effect of MK-4 on trabecular bone loss may result in the maintenance of bone formation and increase in bone turnover assessed by bone histomorphometry. In the present study, from the results of bone histomorphometry in the tibial cortical region (Table 3), it appears that MK-4 inhibited the
reduction in bone formation induced by immobilization. Treatment commenced from 3 wk after the operation to avoid any influence of transition and rapid increase in bone resorption caused immediately by unloading (14). The increase in bone mineral density and strength of the femoral diaphysis may be due to the enhancement of cortical bone formation rather than suppression of bone resorption by MK-4.

Recent epidemiologic studies have implicated vitamin K as a potentially important dietary factor that can affect BMD and the risk of fractures (1, 30, 31). Furthermore, the possible mechanisms whereby suboptimal vitamin K intake and status affect bone metabolism are poorly understood. Natto contains a high concentration of vitamin K2 (MK-7). Yamaguchi and co-investigators reported that the intake of natto increases circulating MK-7 and γ-carboxylated osteocalcin in normal individuals (32). They also reported that both MK-4 and MK-7 accumulated in the femoral bone and may be a useful tool for high-risk subjects with disuse osteopenia. The increase in bone induced by sciatic neurectomy. The increase in bone resorption caused immediately by tenotomy-induced bone resorption in rats. I Bone Miner Res 4: 1201–1208.

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