

Effects of the combination of vitamin K and teriparatide on the bone metabolism in ovariectomized rats

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Abstract. The purpose of the present study was to evaluate the combined effects of vitamin K (VK) and teriparatide (TPTD) on bone mineral density (BMD), mechanical strength and other parameters for bone metabolism using a rat ovariectomized osteoporosis model. Ovariectomized female Sprague-Dawley rats were administered with VK (an oral dose of 30 mg/kg/day), TPTD (a subcutaneous dose of 30 μ g/kg, three times a week) or a combination for 8 weeks. Thereafter, serum levels of γ -carboxylated osteocalcin (Gla-OC) were quantitated by ELISA; BMD and mechanical strength were measured by computed tomography and biomechanical testing, respectively at the femoral metaphysis. Additionally, histomorphometry was performed using the toluidine blue-stained coronal sections of distal femur. The combination of VK and TPTD clearly increased the serum levels of Gla-OC (a specific marker for bone formation) and osteoblast surface (the number of osteoblasts attaching with the surface of cancellous bone), compared to VK or TPTD alone. In addition, the combination of the two agents improved the BMD and bone strength of the femur in the ovariectomized rats, compared to VK or TPTD alone. Taken together, these findings suggest that the treatment with VK and TPTD may have a therapeutic advantage over VK or TPTD monotherapy for postmenopausal osteoporosis, possibly by enhancing the bone formation through the actions on OC and osteoblasts.

Introduction

Postmenopausal osteoporosis is a degenerative disease characterized by reduced bone mass, as estrogen deficiency during menopause enhances the bone turnover, in which bone degradation exceeds bone formation. Postmenopausal osteoporosis leads to bone fragility and a consequent increase in fracture risk. To reduce the risk of fracture, it is important to improve bone strength clinically using several agents, including bisphosphonate, vitamin D, vitamin K (VK) and teriparatide (TPTD). In addition, the combination therapy with these agents can increase the effectiveness of treatment in certain cases and is now the subject of extensive investigation (1,2).

VK is essential for the γ -carboxylation of osteocalcin (OC), a major non-collagenous bone protein, synthesized by osteoblasts. γ -carboxylated OC (Gla-OC) is the most active form and stimulates osteoblasts to enhance mineralization (3,4). Previously, it has been reported that VK binds to the steroid and xenobiotic receptor and modulates the gene transcription in human osteoblastic cells (5,6). Additionally, VK plays roles not only in enhancing bone formation, but also in suppressing bone degradation (7,8). Clinically, VK has been shown to prevent bone loss and fractures in postmenopausal females with osteoporosis (9-12).

By contrast, an intermittent injection of low-dose parathyroid hormone-derived peptide (TPTD) can effectively stimulate bone formation by increasing osteoblast number and activity via the promotion of the differentiation and survival of osteoblasts, thereby increasing trabecular bone mass and strength (13-17). In fact, clinical trials show that TPTD increases bone mineral density (BMD) and decreases the fracture risk in females with postmenopausal osteoporosis (18-20).

The VK-dependent γ -carboxylation of OC was recently reported to enhance the efficacy of TPTD against bone repair in a rat osteotomy model (21). Thus, we hypothesized that the combination of VK and TPTD may improve the bone metabolism in osteoporosis by increasing the bone formation but suppressing the bone degeneration. Therefore, in the present study, the effects of the combination of VK and TPTD on BMD, mechanical strength and other parameters for bone metabolism were examined in a rat ovariectomized osteoporosis model.

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Materials and methods

Animals. All the procedures were carried out according to the Institutional Animal Care and Committee Guide of Juntendo University School of Medicine (Tokyo, Japan). Twenty five female Sprague Dawley rats (13 weeks old; Japan SLC, Tokyo, Japan) were used. The rats were housed in a controlled environment at $25\pm 1^\circ\text{C}$ with a 12-h on/off light cycle and free access to deionized water.

Experiment protocols. After adaptation to the new environment for one week, rats were assigned to five groups: The sham, ovariectomy (OVX), VK, TPTD and VK + TPTD groups. At 14 weeks of age, bilateral OVX was performed from a dorsal approach under inhalation anesthesia of halothane for 20 rats belonging to the OVX, VK, TPTD and VK + TPTD groups. Five rats were subjected to the sham operation in which the ovaries were exteriorized but not removed. VK (30 mg/kg/day) was orally administered to rats in the VK and VK + TPTD groups by feeding with a VK-deficient diet (CLEA Japan, Inc., Tokyo, Japan) containing 65 mg VK (menaquinone MK-4; Eisai Co., Ltd., Tokyo, Japan) and 0.5 g calcium per 100 g diet. Rats in the other groups were fed the VK-deficient diet containing 0.5 g calcium per 100 g diet. Teriparatide acetate (corresponding to the N-terminal 1-34 amino acid residues of human parathyroid hormone consisting of 84 amino acids; Asahi Kasei Pharma Corporation, Tokyo, Japan) was dissolved in saline containing 0.1% rat serum albumin and injected subcutaneously three times a week at a dose of $30\ \mu\text{g}/\text{kg}$ in the TPTD and VK + TPTD groups, and rats in the other groups were injected with saline as a vehicle. After the administration of VK and TPTD for 8 weeks, rats were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg) and blood samples were obtained from the femoral vein. Following sacrifice by exsanguination, bilateral femurs, from which muscles were detached, were collected from every rat. Serum was separated by centrifugation of blood at $770 \times g$ for 20 min at 4°C and stored at -80°C . The right femurs were stored at -80°C for BMD measurement and biomechanical testing; the left femurs were immersed in 70% ethanol solution for histomorphometry.

Biomarker assays. Serum levels of Gla-OC and Glu-OC (the non γ -carboxylated form of OC) were measured using an enzyme-linked immunosorbent assay (Rat Gla-Osteocalcin High Sensitive EIA and Rat Glu-Osteocalcin High Sensitive EIA kits; Takara Bio Inc., Shiga, Japan). Serum levels of C-terminal telopeptide of type I collagen (CTX-I) was measured by an enzyme-linked immunosorbent assay (RatLaps EIA; Immunodiagnostic Systems Ltd., Boldon, UK).

BMD assay. The BMD of femurs was measured by X-ray computed tomography (Latheta LCT-200; Hitachi Aloka Medical, Ltd., Tokyo, Japan). BMD of diaphysis was measured at the mid-diaphysis position of femurs and BMD of metaphysis was measured at 1.5 mm proximal to the growth plate in the condyle of femurs.

Biomechanical testing. Biomechanical properties were measured by a three-point bending test at mid-diaphysis and

a compression test at metaphysis using a servo-hydraulic fatigue-testing instrument (Servopulser; Shimadzu Co., Kyoto, Japan). The mid-diaphysis was placed on the two supports that were 20 mm apart. The load of a three-point bending test was applied in the anteroposterior direction midway between the two supports. Load displacement curves were recorded at a crosshead speed of 6 mm/sec. Alternatively, metaphysis was placed on the test apparatus with the lateral side up. Compression load was applied on the specimens from the lateral aspect to the medial aspect. Load displacement curves were recorded at a crosshead speed of 6 mm/sec. The maximum load, breaking energy and stiffness were calculated using a load-displacement curve.

Histomorphometry. The left femurs were fixed and embedded in glycol methacrylate. Coronal sections of distal femur were cut at a thickness of $20\ \mu\text{m}$ and stained with toluidine blue for histomorphometric analyses. Measurements were performed in the secondary cancellous bone region from 0.2 to 2.25 mm proximal to the growth plate using a semi-automatic image analysis system (OsteoMetrics, Inc., Decatur, GA, USA) and the primary parameters, such as tissue volume (TV), bone volume (BV), bone surface (BS), osteoblast surface (Ob.S) and osteoclast surface (Oc.S), were determined. These data were used to calculate the following parameters: BV/TV, Ob.S/BS and Oc.S/BS.

Statistical analysis. All the data are expressed as mean \pm standard deviation. Multiple comparisons of data among the groups were performed by analysis of variance with Tukey's post hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Biomarker assays. Fig. 1 shows the serum levels of Gla-OC, Glu-OC and CTX-I. The levels of Gla-OC and CTX-I in the OVX group were significantly increased compared to the Sham group ($P < 0.05$), although there was no significant change in the serum level of Glu-OC. Compared to the OVX group, the Gla-OC level was slightly increased in the VK group and significantly increased in the TPTD group ($P < 0.01$) (Fig. 1A). Additionally, the Gla-OC level was further increased in the VK + TPTD group compared to the VK and TPTD groups ($P < 0.05$).

Notably, the Glu-OC level was significantly decreased in the VK group ($P < 0.01$), but increased in the TPTD group ($P < 0.05$) compared to the OVX group (Fig. 1B). The Glu-OC level decreased in the VK + TPTD group compared to the TPTD group, although there was no significant difference between the two groups.

The CTX-I level was decreased in the VK group compared to the OVX group (Fig. 1C). Additionally, the CTX-I level was significantly increased in the TPTD group compared to the Sham group. Of note, the CTX-I level was increased in the TPTD group compared to the VK group but decreased in the VK + TPTD group compared to the TPTD group.

Assay of BMD. Fig. 2 shows the BMD of the femoral metaphysis and diaphysis. The BMD of the femoral metaphysis in the OVX

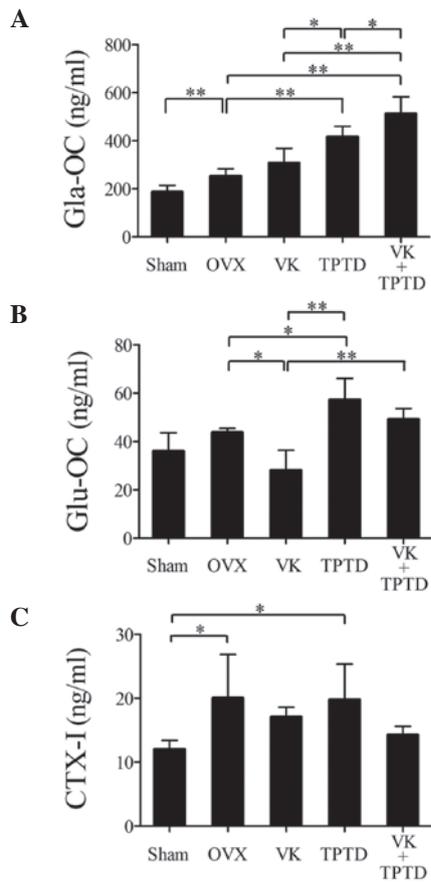


Figure 1. Serum levels of γ -carboxylated osteocalcin (Gla-OC), Glu-OC and C-terminal telopeptide of type I collagen (CTX-I) in rats administered with vitamin K (VK) and teriparatide (TPTD). Rats were assigned to the Sham, ovariectomy (OVX), VK, TPTD and VK + TPTD groups (five rats in each group). Bilateral OVX was performed in rats belonging to the OVX, VK, TPTD and VK + TPTD groups. VK (30 mg/kg/day) was orally administered to rats in the VK and VK + TPTD groups. TPTD was injected subcutaneously three times a week at a dose of 30 μ g/kg in the TPTD and VK + TPTD groups. After the administration of VK and TPTD for 8 weeks, sera were prepared for measurement of (A) Gla-OC, (B) Glu-OC and (C) CTX-I. Data are expressed as mean \pm standard deviation and compared among the groups. * $P < 0.05$ and ** $P < 0.01$.

group was significantly decreased compared to the Sham group ($P < 0.05$) (Fig. 2A). Of note, the decreased level of BMD (OVX group) was not changed by VK administration but significantly increased by TPTD administration ($P < 0.01$). Furthermore, BMD in the combination of VK and TPTD group was significantly increased compared to the VK group ($P < 0.01$) and increased compared to the TPTD group. By contrast, BMD of the femoral diaphysis was not significantly different among the five groups (Sham, OVX, VK, TPTD and VK + TPTD) (Fig. 2B).

Biomechanical testing. Fig. 3 shows the results of biomechanical testing of metaphysis. The maximum load and breaking energy in the OVX group were lower compared to the Sham group (Fig. 3A and B). Notably, the maximum load and breaking energy in the TPTD group were increased compared to the OVX group and breaking energy in the VK group was increased compared to the OVX group (Fig. 3A and B). Additionally, in the VK + TPTD group, the maximum load and breaking energy were significantly increased compared to the OVX group ($P < 0.05$).

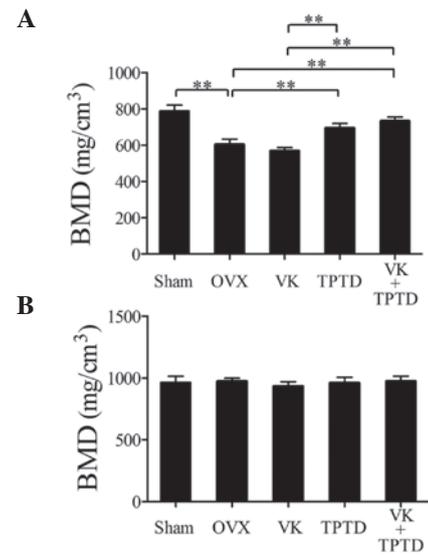


Figure 2. Bone mineral density (BMD) of the femoral metaphysis and diaphysis of rats administered with vitamin K (VK) and teriparatide (TPTD). After the administration of VK and TPTD for 8 weeks, BMD was measured using the (A) femoral metaphysis and (B) diaphysis of rats belonging to the Sham, ovariectomy (OVX), VK, TPTD and VK + TPTD groups (five rats in each group). Data are expressed as mean \pm standard deviation and compared among the groups. ** $P < 0.01$.

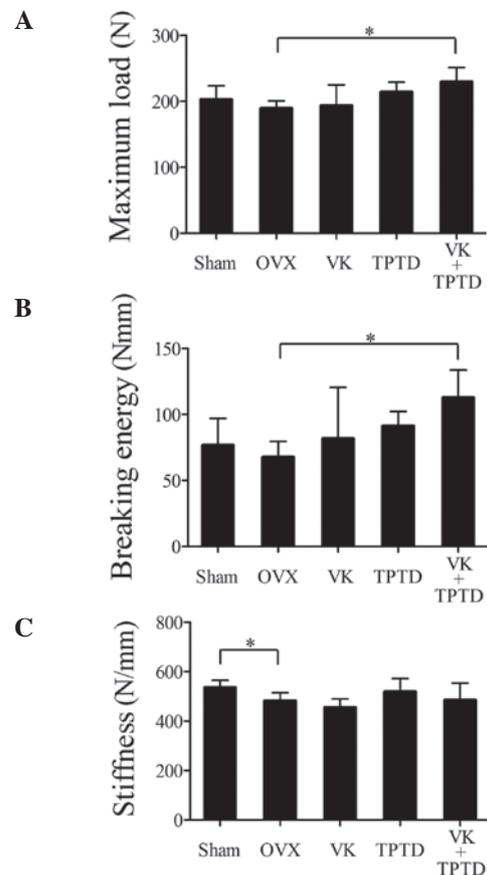


Figure 3. Biomechanical properties of the femoral metaphysis in rats administered with vitamin K (VK) and teriparatide (TPTD). After the administration of VK and TPTD for 8 weeks, the biomechanical properties of (A) maximum load, (B) breaking energy and (C) stiffness were measured by a compression test at the femoral metaphysis of rats belonging to the Sham, ovariectomy (OVX), VK, TPTD and VK + TPTD groups (five rats in each group). Data are expressed as mean \pm standard deviation and compared among the groups. * $P < 0.05$.

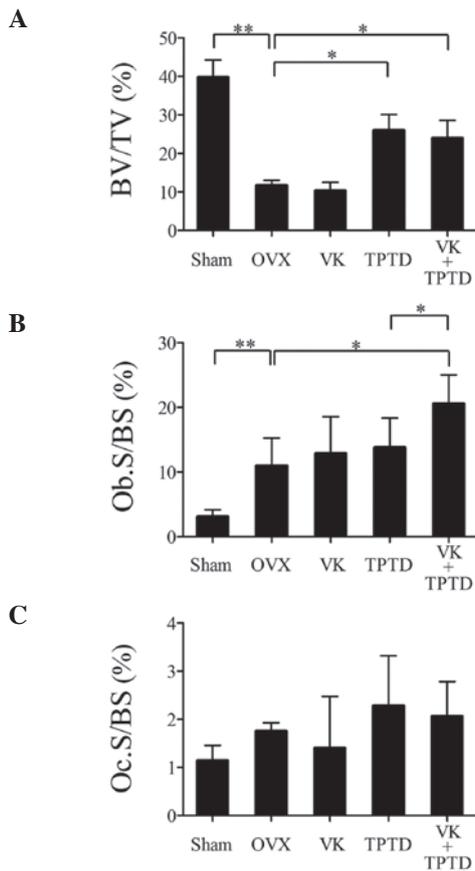


Figure 4. Histomorphometry of the femoral metaphysis in rats administered with vitamin K (VK) and teriparatide (TPTD). Histomorphometry of (A) bone volume (BV)/tissue volume (TV), (B) osteoblast surface (Ob.S)/bone surface (BS) and (C) osteoclast surface (Oc.S)/BS were measured at the femoral metaphysis of rats belonging to the Sham, ovariectomy (OVX), VK, TPTD and VK + TPTD groups (five rats in each group). Data are expressed as mean \pm standard deviation and compared among the groups. * $P < 0.05$ and ** $P < 0.01$.

The stiffness of the metaphysis was significantly decreased in the OVX group compared to the Sham group ($P < 0.05$) (Fig. 3C). The decreased level of the stiffness was further decreased in the VK group but increased in the TPTD group compared to the OVX group (Fig. 3C). Notably, the increased level of stiffness in the TPTD group was decreased by the combination with VK (the VK + TPTD group).

Similar to the finding in BMD (Fig. 2B), the biomechanical parameters of femoral diaphysis (maximum load, breaking energy and stiffness) were not significantly different among the five groups (Sham, OVX, VK, TPTD and VK + TPTD) (data not shown).

Bone histomorphometry. Fig. 4 shows the results of bone histomorphometric analysis of femoral metaphysis. BV/TV was significantly decreased in the OVX compared to the Sham group ($P < 0.01$) (Fig. 4A). The decreased level of BV/TV was significantly increased in the TPTD group ($P < 0.05$), but not changed in the VK group. In addition, the decreased level of BV/TV was significantly increased in the VK + TPTD group compared to the OVX group ($P < 0.05$), although there was no significant difference between the TPTD and VK + TPTD groups.

Ob.S/BS was significantly increased in the OVX group compared to the Sham group (Fig. 4B). Additionally, Ob.S/BS was increased in the VK and TPTD groups compared to the OVX group. Notably, Ob.S/BS was significantly increased in the VK + TPTD group compared to the OVX group and TPTD group ($P < 0.05$).

Oc.S/BS was slightly increased in the OVX group compared to the Sham group (Fig. 4C). Of note, Oc.S/BS was decreased in the VK group compared to the OVX group but increased in the TPTD group compared to the OVX group. Notably, Oc.S/BS was decreased in the VK + TPTD group compared to the TPTD group.

Discussion

In the present study, the effects of a combination of VK and TPTD on BMD, mechanical strength and other parameters were examined for bone metabolism using a rat ovariectomized osteoporosis model. The combination of VK and TPTD markedly increased the serum levels of Gla-OC (a specific marker for bone formation) and Ob.S (the number of osteoblasts attaching with the surface of cancellous bone) compared to VK or TPTD. In addition, the combination of VK and TPTD improved the BMD and bone strength of the femur in the estrogen-deficient (ovariectomized) osteoporosis of rats. These findings suggest that the treatment with VK and TPTD may have a therapeutic advantage over VK or TPTD monotherapy for postmenopausal osteoporosis, possibly by enhancing the bone formation through the increase of OC and the activation of osteoblasts.

Ovariectomized animals have been widely used as an experimental model of osteoporosis. This model is highly reproducible and characterized by loss of bone mass due to the high bone turnover, in which the bone formation and degradation are enhanced due to estrogen deficiency (22). In the present study, OVX caused the loss of mineral and mechanical properties in the metaphysis of femurs, but did not affect these parameters in the diaphysis of femurs (Figs. 2 and 3). Consistent with this observation, OVX reportedly shows the loss of cancellous (metaphysis) bone compared to cortical (diaphysis) bone (23). In addition, the present study identified that OVX induced a significant increase in the serum levels of Gla-OC (a bone formation parameter) and CTX-I (a bone degradation parameter), confirming the enhanced bone turnover in the model following estrogen deficiency.

Administration of VK increased Gla-OC and decreased CTX-I in sera. Similarly, VK modulated Ob.S/BS and Oc.S/BS in the histomorphometric parameters. Although these changes were small, the present results suggest that VK increases the bone formation but suppresses the bone degradation in an osteoporosis model, as previously reported for the effect of VK (7,8,24,25). Of note, VK administration significantly decreases Glu-OC but increases Gla-OC in sera, possibly by acting on the OC metabolism. Consistent with this, it is reported that non- or undercarboxylated osteocalcin (Glu-OC) is decreased, but Gla-OC is increased in sera of individuals administered with VK (9).

TPTD effectively promotes bone formation and enhances cancellous bone mass by stimulating the number and activity of trabecular osteoblasts in ovariectomized rats (26-28). The present study showed that the administration of TPTD

promotes the bone formation as indicated by increasing Gla-OC in sera and Ob.S/BS in the histomorphometry. In addition, TPTD administration increased not only Gla-OC but also Glu-OC in sera. Thus, it is possible that TPTD enhances the synthesis of OC itself via the stimulation of osteoblasts, thereby increasing Glu-OC. The effects of TPTD on bone degradation are unclearly defined; however, the present study has indicated that TPTD increased Oc.S/BS in histomorphometry but did not change CTX-I level in sera.

The combination administration of VK and TPTD increased Gla-OC and Ob.S/BS compared to TPTD treatment alone. By contrast, the combination administration reduced the increased levels of CTX-I compared to TPTD treatment alone. These results suggest that the combination of VK and TPTD promotes the bone formation but suppresses bone degradation. Of note, VK reduced the CTX-I and Oc.S/BS compared to OVX and the combination of VK and TPTD decreased these parameters compared to TPTD alone (Figs. 1 and 4). These results suggest that VK suppresses the bone degradation when administered alone or in combination with TPTD.

In the present study, TPTD administration significantly increased the BMD and slightly enhanced the bone strength (maximum load and breaking energy). These results are consistent with previous studies showing that TPTD restores lost bone mass and enhances bone strength in ovariectomized rat models (29-32). By contrast, VK administration increased the breaking energy but did not increase the BMD. Consistently, previous studies indicated that VK improves bone strength without necessarily increasing BMD (33). These effects of VK are considered to be due to the increase of active (carboxylated) OC and lysyl oxidase-mediated enzymatic cross-linking of collagen, thereby improving bone strength (elasticity and toughness) without significantly affecting BMD (34-36). Notably, the combination of VK and TPTD increased the BMD compared to VK alone. In addition, the combination further increased bone strength (breaking energy and maximum load) compared to VK or TPTD alone. These results suggest that the combination of VK and TPTD is effective in increasing not only BMD but also bone strength. Notably, the administration of VK or the combination of VK and TPTD decreased the stiffness compared to OVX or TPTD alone, respectively. These observations may suggest that VK possibly makes the cancellous bone pliable. Thus, the combination of VK and TPTD is expected to improve not only the bone strength, but also the bone quality.

In conclusion, the present study revealed that the combination of VK and TPTD promoted the bone formation and improved the BMD and bone strength of the femurs in ovariectomized rats. Thus, the combination administered is expected to be effective in preventing fractures in postmenopausal osteoporosis. Further studies are required to clarify the molecular mechanisms for the cooperative action of VK and TPTD on the bone metabolism in the postmenopausal osteoporosis.

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