

Effects of the combined administration of risedronate and menatetrenone on bone loss induced by tacrolimus in rats

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SUMMARY Tacrolimus, a calcineurin inhibitor, affects bone metabolism and increases the risk of fracture due to marked bone loss. Bisphosphonates increase the bone mineral density (BMD) in osteoporosis patients. Menatetrenone has less positive effects on BMD but reduces the risk of fracture by improving bone quality. In this study, we investigated the effectiveness of the combined administration of risedronate and menatetrenone against bone loss induced by tacrolimus. Wistar rats were divided into four groups: [1] control, [2] tacrolimus at 1.5 mg/kg, [3] tacrolimus + risedronate at 1.0 mg/kg, and [4] tacrolimus + risedronate + menatetrenone at 20 mg/kg. After the drugs were administered for 4 weeks, bone histomorphometric analysis was performed and bone strength was evaluated using a three-point bending method. BMD was measured using quantitative computed tomography. Tacrolimus significantly reduced the BMD and strength properties of the lower limb bones. These tacrolimus-induced decreases were suppressed by risedronate treatment. The combined administration of risedronate and menatetrenone more significantly improved bone strength properties than risedronate alone. Bone histomorphometric analysis revealed a significant increase in bone resorption with tacrolimus. Risedronate alone significantly suppressed the tacrolimus-induced increase in bone resorption but simultaneously reduced bone formation. On the other hand, the combined administration of risedronate and menatetrenone suppressed the tacrolimus-induced increase in bone resorption, in addition to the significant risedronate-induced decrease in bone formation. This study suggests that the combined administration of risedronate and menatetrenone improves bone strength in tacrolimus-treated rats by preventing and ameliorating the risedronate-induced suppression of bone formation.

Keywords Bisphosphonate, menatetrenone, combined administration, bone loss, tacrolimus

1. Introduction

To further extend the healthy life expectancy in the super-aged societies of developed countries, fractures, which significantly reduce quality of life and vital prognosis in patients, should be prevented. Bone is a metabolically active organ that undergoes continuous remodeling, consisting of bone resorption by osteoclasts and bone formation by osteoblasts (1). An imbalance of bone remodeling induced skeletal disorders, such as osteopenia or osteoporosis (2,3). Aging and menopause are the main causes of bone fragility leading to fractures, whereas certain diseases and drugs significantly affect bone metabolism, resulting in an increased risk of fracture. We previously reported that tacrolimus, a

calcineurin inhibitor, increased osteoclast activity, which regulates bone metabolism, and induced bone loss (4). To promote immunosuppressive therapy, which is essential for the prevention of rejection in recipients, prevention and treatment methods should be established using pharmacotherapy for tacrolimus-induced bone loss.

Bone strength is defined by bone mineral density (BMD) and bone quality, which refers to bone microstructure, bone turnover, micro-damage, and bone mineralization (5). Bisphosphonates, bone resorption inhibitors, are used worldwide for the treatment of osteoporosis. Bisphosphonates suppress bone resorption by inhibiting osteoclast activity (6,7). Of note, bisphosphonates have been demonstrated to increase

BMD in clinical studies (8-11) and osteoporosis animal models (12-14). However, long-term administration of bisphosphonates may degrade bone strength by markedly suppressing bone remodeling (15-17) and increase the risk of developing atypical femoral fractures (18-21).

Menatetrenone, vitamin K₂, acts as an essential coenzyme for the γ -carboxylation of glutamate residues in osteocalcin, a bone matrix protein (22,23), and significantly reduces the risk of fracture (24,25). As menatetrenone slightly increases BMD (26), its ability to reduce the risk of fracture is primarily explained by improved bone quality (27). Therefore, the combined administration of bisphosphonate and menatetrenone, which have different mechanisms of action on bone metabolism, may have beneficial effects on both BMD and bone quality, which determine bone strength, although its detailed effects remain unclear. In the present study, we investigated the effects of the combined administration of risedronate and menatetrenone on tacrolimus-induced bone loss in rats.

2. Materials and Methods

2.1. Animals

Four week-old male Wistar rats weighing 70-80 g were purchased from CLEA Japan Inc. (Tokyo, Japan). Animals were housed at $22 \pm 2^\circ\text{C}$ and $55 \pm 5\%$ humidity on a 12-h light-dark cycle with *ad libitum* access to standard chow (MF; Oriental Yeast Co., Tokyo, Japan) and water. All procedures were approved by the Animal Research Committee of Niigata University of Pharmacy and Applied Life Sciences in accordance with the Japanese Government Animal Protection and Management Law, Japanese Government Notification on Feeding and Safekeeping of Animals.

2.2. Drugs

Commercially available tacrolimus (Astellas Pharma Inc., Tokyo, Japan) and risedronate (Eisai Co., Ltd., Tokyo, Japan) agents were obtained suspended in a 0.2% carboxymethylcellulose sodium solution (CMC-Na; Sigma-Aldrich, St. Louis, MO, USA). Menatetrenone (Eisai Co., Ltd., Tokyo, Japan) was suspended in olive oil.

2.3. Experimental procedure

Animals were randomly divided into four groups (10 animals/group): [1] control treated with the vehicle (0.2% CMC-Na), [2] tacrolimus at 1.5 mg/kg, [3] tacrolimus at 1.5 mg/kg + risedronate at 1.0 mg/kg, and [4] tacrolimus at 1.5 mg/kg + risedronate at 1.0 mg/kg + menatetrenone at 20 mg/kg. Drug doses were selected based on previous reports relevant to tacrolimus (4),

risedronate (12), and menatetrenone (27). Drugs were administered *via* oral gavage in a volume of 0.1 mL/100 g of body weight once daily for 4 weeks. All animals were euthanized under CO₂ anesthesia 24 h after the final drug was administered. The femur and tibia were dissected, and soft tissue was removed.

2.4. Bone strength analysis

Bone strength of the femoral mid-diaphysis was evaluated *via* a three-point bending method using a mechanical testing machine (EZ-S; Shimadzu, Tokyo, Japan). The femur was positioned on two supports placed 10 mm apart. The bending load was vertically applied to the mid-diaphysis with a crosshead speed of 1.0 mm/min until fracture. The load deformation curves were calculated using operation software (Trapezium X; Shimadzu, Tokyo, Japan), and the maximum load, breaking energy, and stiffness were directly calculated from the load deformation curve.

2.5. BMD measurements

BMD of the whole femur and tibia was measured using quantitative computed tomography (LaTheta LCT-100; Aloka, Tokyo, Japan) with a pixel size of $250 \times 250 \mu\text{m}$ and slice thickness of 1 mm. The values of BMD were calculated using LaTheta software (ver. 1.31; Aloka, Tokyo, Japan).

2.6. Bone histomorphometry

We prepared non-decalcified specimens from the proximal tibia metaphysis according to the following method: The tibia was fixed with 70% ethanol for 7 days, stained with Villanueva Bone Stain (basic fuchsin, fast green, orange G, and azure II; Merck, Darmstadt, Germany) in 70% methanol for 7 days, and embedded in a methyl methacrylate resin. The resin blocks were then sliced to 5- μm thickness on a microtome (Leica RM2255; Leica Inc., Nussloch, Germany). All bone histomorphometric parameters were measured at the secondary spongiosa region. To exclude the primary spongiosa, the measurement region was 0.42.1 mm distal to the lowest point of the growth plate and 0.2 mm from the lateral cortex.

Bone histomorphometric measurements were performed using a semiautomatic image analyzing system (Histometry RT CAMERA; System Supply, Nagano, Japan) with $\times 400$ magnification. Bone structural parameters obtained included bone volume per tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), and trabecular separation (Tb.Sp). Bone formation parameters included the osteoblast surface per bone surface (Ob.S/BS). Bone resorption parameters included the eroded surface per bone surface (ES/BS), osteoclast surface per bone surface (Oc.S/BS),

and osteoclast number per bone surface (N.Oc/BS).

The dynamic parameter was measured using a double fluorescent labeling technique. For labeling, all rats were injected subcutaneously with 25 mg/kg of tetracycline (Sigma-Aldrich, St. Louis, MO, USA) and 10 mg/kg of calcein (Wako Pure Chemical Industries, Osaka, Japan) 5 and 2 days before they were euthanized, respectively. The labeled surface that reflected the calcification front at the time of tetracycline and calcein administration was visualized using a fluorescent microscope (Olympus BX50; Olympus America Inc., Center Valley, PA, USA). The parameters of single- and double-labeled surface (sLS and dLS) and inter-label thickness, and times (Ir.L.Th and Ir.L.t) were used in the calculation of the mineralizing surface per bone surface (MS [dLS+sLS/2]/BS), mineral apposition rate (MAR; Ir.L.Th/Ir.L.t).

Figure 1 shows the scheme of the primary parameters (28). Standard bone histomorphometrical nomenclature, symbols, and units were based on those described in the report of the American Society for Bone and Mineral Research Histomorphometry Nomenclature Committee (29).

2.7. Statistical analysis

Data are presented as the mean \pm standard error (SE). Differences between groups were analyzed by one-way ANOVA followed by Dunnett's multiple comparisons. $p < 0.05$ was considered significant.

3. Results

3.1. Bone strength properties

The maximum load and breaking energy in the tacrolimus group were significantly reduced (37% and 40%, respectively) compared with those in the control group (Figure 2). In addition, the maximum load and breaking energy in the risedronate and risedronate + menatetrenone groups were significantly higher

than those in the tacrolimus group. Furthermore, the maximum load and breaking energy in the risedronate + menatetrenone group were significantly higher than those in the risedronate group.

3.2. BMD

Femoral and tibial BMD were significantly reduced (9% and 11%, respectively) in the tacrolimus group compared with those in the control group (Figure 3). The BMD of femur and tibia in the risedronate and risedronate + menatetrenone groups were significantly higher than those in the tacrolimus group. On the other hand, the BMD of femur and tibia in the risedronate + menatetrenone group were comparable with and not significantly different from those in the risedronate group.

3.3. Bone histomorphometric evaluation

Trabecular bone structural parameters according to the bone histomorphometry of the proximal tibia metaphysis are shown in Figure 4. Tacrolimus treatment significantly reduced the BV/TV, Tb.Th, and Tb.N (50%, 20%, and 66%, respectively), and increased the Tb.SP (57%) relative to those in the control group. These bone structural parameters in the risedronate and risedronate + menatetrenone groups were significantly higher than those in the tacrolimus group.

Bone formation parameters, Ob.S/BS, MS/BS, and MAR, in the tacrolimus group did not significantly differ from those in the control group, whereas bone resorption parameters, ES/BS, Oc.S/BS, and N.Oc/BS (69%, 70%, and 87%, respectively), were significantly increased (Figure 5). The tacrolimus-induced increases in ES/BS, Oc.S/BS, and N.Oc/BS were significantly suppressed by risedronate alone or risedronate + menatetrenone. However, Ob.S/BS, MS/BS, and MAR (83%, 70%, and 54%, respectively), were significantly reduced in the risedronate group compared with the other groups. In contrast, Ob.S/BS, MS/BS, and MAR were significantly increased in the risedronate + menatetrenone group compared with those in the risedronate group, and were comparable with those in the control group.

Typical fluorescence microphotographs of the slices assessed by bone histomorphometry are shown in Figure 6. These images confirmed the marked decrease in fluorescence labeled surface with tetracycline and calcein in the risedronate group compared with that in the control group. In contrast, bones in the risedronate + menatetrenone group had a marked increase in fluorescence labeled surface and inter-label thickness compared with those in the risedronate group.

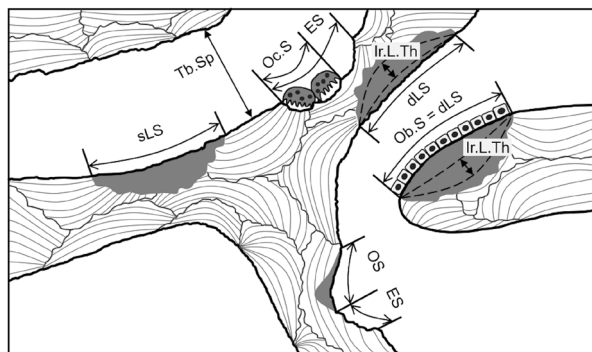


Figure 1. The scheme of the primary bone histomorphometric parameters. Tb.Sp; trabecular separation, dLS; double-labeled surface, sLS; single-labeled surface, Ir.L.Th; inter-labels thickness, Ob.S; osteoblast surface, OS; osteoid surface, ES; eroded surface, Oc.S; osteoclast surface.

4. Discussion

The present study examined the effects of the combined

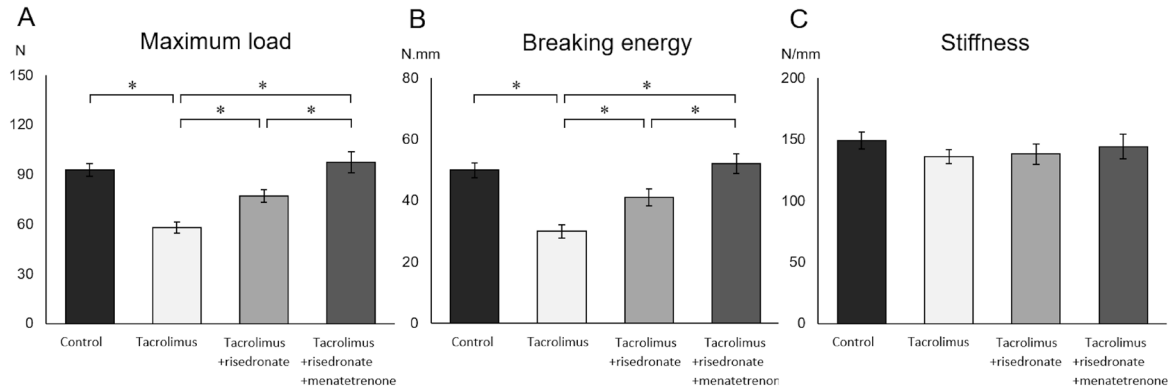


Figure 2. Bone strength properties (A: maximum load, B: breaking energy, C: stiffness) of the femoral mid-diaphysis in each group. Bone strength was evaluated by a three-point bending method. Data represents the mean ± SE (n = 10). *p < 0.05.

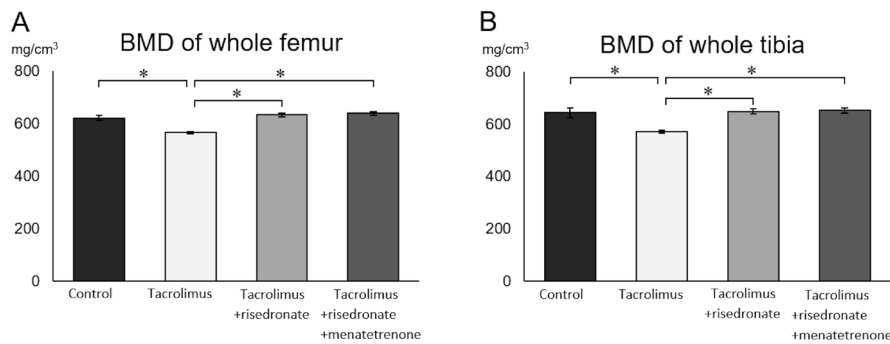


Figure 3. BMD of whole femur (A) and tibia (B) in each group. BMD was measured using quantitative computed tomography. Data represents the mean ± SE (n = 10). *p < 0.05.

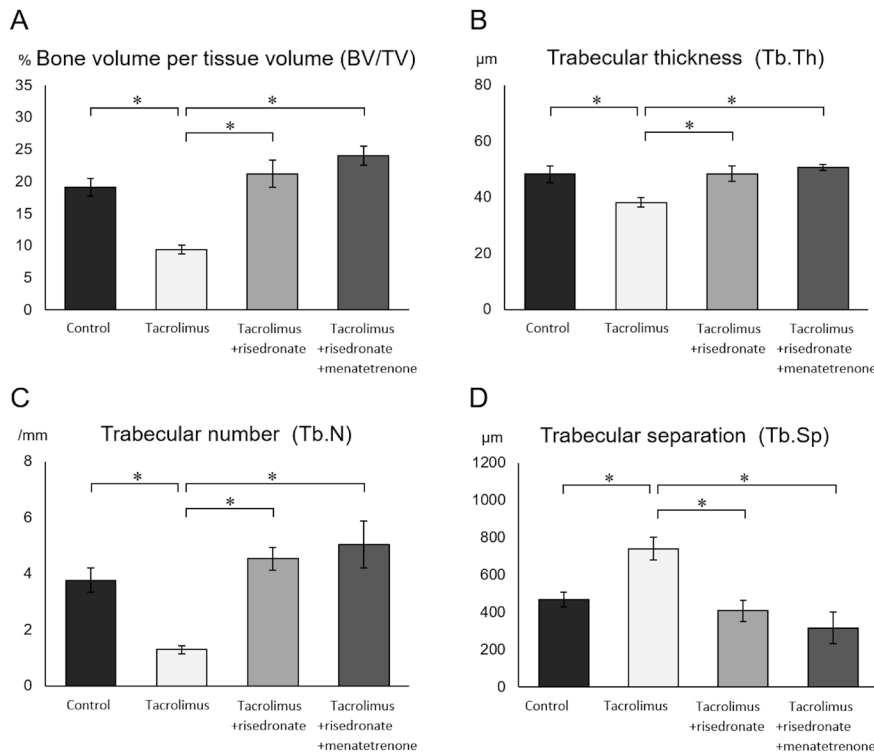


Figure 4. Trabecular bone structural parameters (A: bone volume per tissue volume (BV/TV), B: trabecular thickness (Tb.Th), C: trabecular number (Tb.N), D: trabecular separation (Tb.Sp)) according to the bone histomorphometry of the proximal tibia metaphysis. Data represents the mean ± SE (n = 10). *p < 0.05.

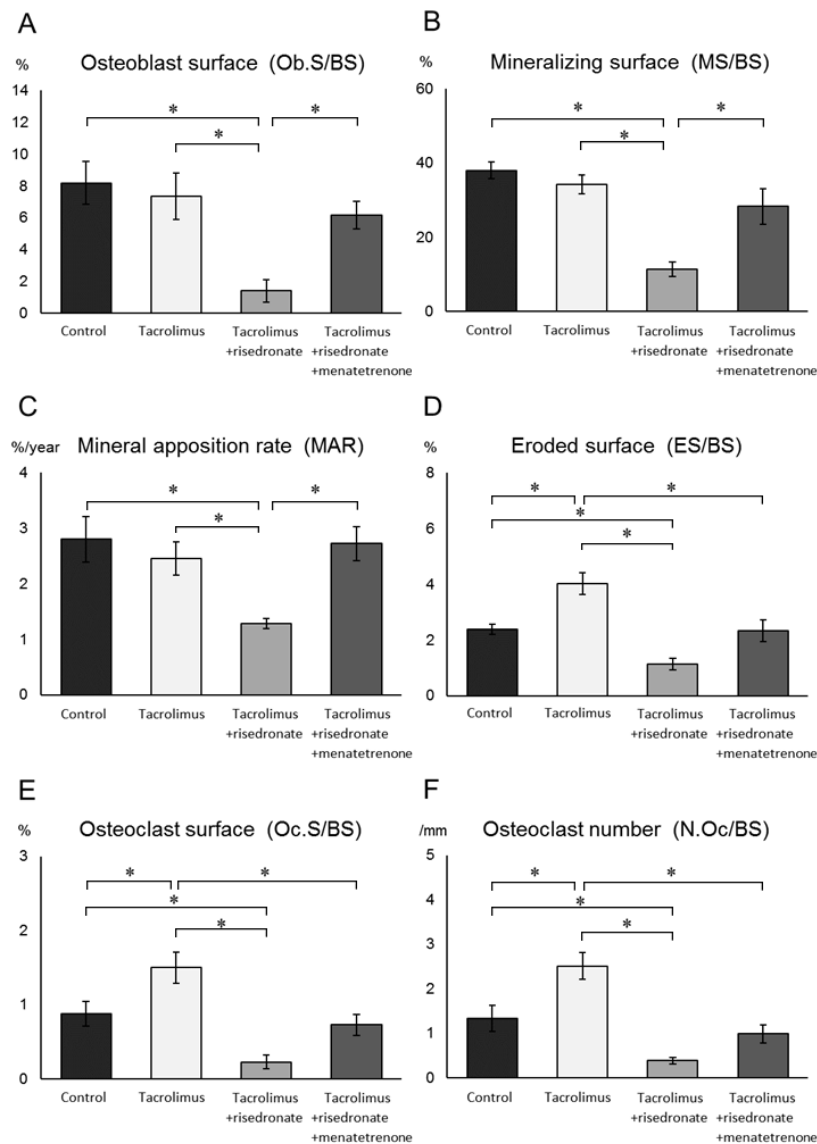


Figure 5. Bone formation parameters (A: osteoblast surface (Ob.S/BS), B: mineralizing surface (MS/BS), C: mineral apposition rate (MAR), D: eroded surface (ES/BS), E: osteoclast surface (Oc.S/BS), F: osteoclast number (N.Oc/BS)) according to bone histomorphometry of the proximal tibia metaphysis. Data represents the mean \pm SE ($n = 10$). * $p < 0.05$.

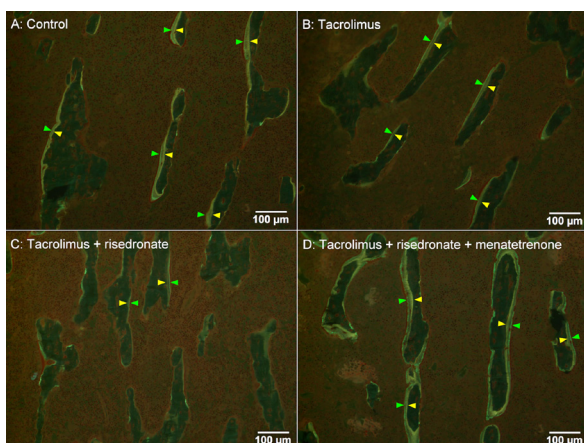


Figure 6. Typical micrographs of the slices assessed by bone histomorphometry under fluorescence. The labeling surface with tetracycline and calcein is indicated by the yellow arrows and the green arrows, respectively.

administration of risedronate and menatetrenone on tacrolimus-induced bone loss. The administration of tacrolimus at 1.5 mg/kg/day for 4 weeks significantly reduced the femoral and tibial BMD in rats. The tacrolimus-induced decreases in the BMD of the lower limb bones were significantly suppressed by risedronate alone, although no additive effects were achieved by the combined administration of risedronate and menatetrenone. On the other hand, the three-point bending test of bone strength properties demonstrated that risedronate was more effective in preventing and ameliorating the tacrolimus-induced decrease in bone strength properties when combined with menatetrenone than when administered alone.

Only a few studies have examined the effects of anti-osteoporosis drugs on tacrolimus-induced bone loss. The clinical studies (30,31) and animal

studies (32,33) found significant protective effects of bisphosphonate on calcineurin inhibitor-induced bone loss. However, long-term administration of bisphosphonate increases microdamage accumulation in the bone (15-17) and is involved in the development of atypical femoral fractures (18-21). Specifically, the marked suppression of bone turnover by long-term bisphosphonate administration may degrade bone quality, resulting in bone fragility. To prevent the bone damage induced by immunosuppressive agents, care should be exercised not only to increase BMD, but also to improve bone quality.

Several studies using osteoporosis animal models also reported the positive effects of the combined administration of bisphosphonate and menatetrenone on bone metabolism (34,35). Menatetrenone is expected to reduce the risk of fracture primarily by improving bone quality (27) because of its negligible bone mass-increasing effects (26). Menatetrenone may improve the degradation of bone quality induced by bisphosphonate. Therefore, in the present study, in order to evaluate the bone quality, we performed a bone histomorphometry of the proximal tibia metaphysis. Bone histomorphometric analysis revealed the impaired microstructure of trabecular bone in rats administered tacrolimus. It also significantly increased the ES/BS, Oc.S/BS, and N.Oc/BS without significantly affecting bone formation. Thus, as previously reported (4), tacrolimus caused rarefaction of the trabecular bone by increasing bone resorption. Risedronate alone significantly suppressed the tacrolimus-induced increase in bone resorption and significantly reduced the bone formation parameters, Ob.S/BS, MS/BS, and MAR. MS/BS and MAR are considered an index of osteoblast differentiation and proliferation (36). Of note, Ob.S/BS, MS/BS, and MAR were significantly higher in the risedronate + menatetrenone group than in the control group. Thus, the suppression of bone formation by risedronate was significantly prevented and ameliorated by the combined administration of risedronate and menatetrenone. Menatetrenone promotes osteocalcin production and mineralization in osteoblasts (37,38), and suppresses osteoclast formation and bone resorption *in vitro* (39). The additive improving effects of the combined administration of risedronate and menatetrenone on the tacrolimus-induced decrease in bone strength may be partially explained by the menatetrenone-induced enhancement of bone formation.

In conclusion, the present study investigated the effectiveness of the combined administration of risedronate and menatetrenone against tacrolimus-induced bone loss. Risedronate alone and risedronate + menatetrenone significantly improved tacrolimus-induced decreases in bone strength properties and BMD. Moreover, the combined administration of risedronate and menatetrenone was highly effective in improving the tacrolimus-induced decrease in bone

strength properties. The effectiveness of the combined administration of anti-osteoporosis drugs with different mechanisms of action may be caused by menatetrenone, which ameliorated the marked suppression of bone formation due to bisphosphonates.

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