

Prior treatment with vitamin K₂ significantly improves the efficacy of risedronate

Y. Matsumoto · Y. Mikuni-Takagaki · Y. Kozai ·
K. Miyagawa · K. Naruse · H. Wakao · R. Kawamata ·
I. Kashima · T. Sakurai

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Abstract

Summary Prior 8-week treatment with menatetrenone, MK-4, followed by 8-week risedronate prevented the shortcomings of individual drugs and significantly increased the strength of ovariectomized ICR mouse femur compared to the ovariectomized (OVX) controls. Neither MK-4 following risedronate nor the concomitant administration may be recommended because they brought the least beneficial effect.

Introduction The objective of this study was to determine the best combinatory administration of risedronate at 0.25 mg/kg/day (R) with vitamin K₂ at approximately 100 µg MK-4/kg/day (K) to improve strength of osteoporotic mouse bone.

Methods Thirteen-week-old ICR mice, ovariectomized at 9-week, were treated for 8 weeks with R, K, or R plus K (R/K), and then, either the treatment was withdrawn (WO) or switched to K or R in the case of R and K. After another 8 weeks, the mice were killed, and mechanical tests and analyses of femur properties by peripheral quantitative computed tomography, microfocus X-ray tube computed tomography, and confocal laser Raman microspectroscopy were carried out.

Results The K to R femur turned out superior in parameters tested such as material properties, bone mineral density, BMC, trabecular structure, and geometry of the cortex. The increased cross-sectional moment of inertia, which occurred after K withdrawal, was prevented by risedronate in K to R. In addition to K to R, some properties of R to WO diaphysis and K to WO epiphysis were significantly better than OVX controls.

Conclusion Prior treatment with MK-4 followed by risedronate significantly increased femur strength in comparison to the OVX controls.

Y. Matsumoto · Y. Kozai · H. Wakao · R. Kawamata ·
I. Kashima · T. Sakurai (✉)
Division of Radiology, Department of Maxillofacial
Diagnostic Science, Kanagawa Dental College,
82 Inaokacho,
Yokosuka, Kanagawa 238-8580, Japan
e-mail: sakurait@kdcnet.ac.jp

Y. Mikuni-Takagaki (✉) · K. Miyagawa
Department of Functional Biology, Kanagawa Dental College,
82 Inaokacho,
Yokosuka, 238-8580 Kanagawa, Japan
e-mail: yukomtak@kdcnet.ac.jp

K. Naruse
Department of Orthopedic Surgery,
Kitasato University School of Medicine,
Tokyo, Japan

Present address:
K. Miyagawa
Department of Craniofacial Developmental Biology,
Osaka University Graduate School of Dentistry,
Suita, Osaka, Japan

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Introduction

Bisphosphonate is one of the most effective drugs currently available for suppressing bone resorption. Naturally, combination therapies with other antiresorptive or formative agents have been investigated: PTH [1–3], vitamin D [2, 4], estrogen [5–7], and other agents [8]. Risedronate, a pyridinyl (amino) bisphosphonate, significantly reduces

the risk of hip fracture among elderly women with confirmed osteoporosis and if combined with estrogen or raloxifene, produces greater gains in bone mass in comparison to single-agent treatment [9]. Oral administration or intake from food of vitamin K₂, on the other hand, has been shown to prevent the occurrence of fractures in Japanese women [10, 11] and was reported to prevent bone loss partly through the improved bone formation in animal studies [12]. It was also reported that vitamin K₂ (MK-4) inhibited bone resorption [13]. A more recent cellular study reported that the osteoprotective action of vitamin K₂ is through steroid and xenobiotic receptor (SXR)/pregnane X receptor (PXR)-modulated Msx2 gene transcription [14]. Such an entirely different pharmacological action of vitamin K₂ from other drugs would make it worth studying combinatory administration with bisphosphonate. Limited reports of trabecular bone implied that the combined treatment is more efficacious in osteoporotic rats [15, 16], while others have reported otherwise [17, 18]. Therefore, the efficacy of their combinatory use was further investigated in ovariectomized (OVX) ICR mice to clarify the effect on the cortical bone and strength. We tried to separate the effect of vitamin K₂ on matrix from that on mineral and to compare with the effect of risedronate by lowering the experimental vitamin K₂ intake level to ~100 µg MK-4/kg/day, which is at the dietary level.

Materials and methods

Experimental animals

The Animal Care Committee of Kanagawa Dental College approved the entire experimental protocol. Nine-week-old ICR mice were purchased from Japan Clea (Tokyo, Japan). All animals were kept under local vivarium conditions (temperature 23.3°C, humidity 55% and a 12-h on/off light cycle).

Sixteen-week-treatment experiment

Fifty-nine, 9-week-old, female ICR mice were either ovariectomized ($n = 43$) or sham-operated ($n = 8$). After a month, during which all mice were fed with conventional rodent food pellets, the ovariectomized mice were divided into six groups. In addition to the OVX group ($n = 8$), five groups ($n = 7$) received medication, which was switched at the 8-week midpoint. In the K to R group, mice were treated with MK-4 for 8 weeks and then with risedronate for eight more weeks. R to K mice were treated with risedronate first and then with vitamin K₂. K to WO, R to WO, and R/K to WO mice received either vitamin K₂, risedronate, or both for 8 weeks, and then the drug(s) was withdrawn. Except in the OVX

groups during K and R/K period, which received pellets containing 50 µg/100 g vitamin K₂ (MK-4), all animals received the conventional rodent food. Both the conventional rodent pellets (CE-2) and the vitamin K₂ pellets were prepared by Japan Clea with MK-4 kindly provided by Eizai (Tokyo, Japan). Calculated from the average 6 to 7 g a day consumption of the ration, the pellets were prepared so that the animals received ~100 µg MK-4/kg/day, which is at a dietary level. During the R or R/K period, mice received 0.25 mg/kg of daily oral risedronate after 2-h fasting. They were fed after another 2-h fasting. The femurs were excised from mice euthanized after the 16-week therapeutic period and were preserved at -80°C for microfocused X-ray computed tomography (micro-CT) and peripheral quantitative computed tomography (pQCT) analyses and confocal Raman spectroscopy.

Eight-week-midpoint experiment

To examine femurs at the 8-week midpoint, total of 42 ICR mice were treated under similar conditions to those of the 16-week experimental animals. They underwent either sham surgery ($n = 9$) or an ovariectomy ($n = 33$). OVX groups include control OVX (OVX, $n = 9$), OVX treated with risedronate (OVX-R, $n = 8$) or vitamin K₂ (OVX-K, $n = 8$), and the concomitant administration (OVX-R/K, $n = 8$).

Microfocused X-ray computed tomography

Using MCT-CB 130F (Hitachi Medico, Tokyo, Japan), three-dimensional imaging data of the distal epiphyseal region of the femur, between 1.5 to 2.75 mm proximal to the growth plate, were obtained. The spatial resolution was set to 7 µm with the voxel size of 17.8 × 17.8 × 17.8 (µm), and the tube voltage and current were 60 kV and 100 µA, respectively. The resolution was set to medium (200 projections each), and slice thickness and increment were set to 20 µm. A morphological analysis was carried out using TRI 3D BONE (Ratoc System Engineering, Tokyo) for such parameters as BV (mm³), bone volume; BS (mm²), bone surface; BV/TV (%), bone volume fraction; Tb.Th (µm), trabecular thickness; Tb.N (1/mm), trabecular number; Tb.Sp (µm), trabecular separation; Tb.Spac (µm), trabecular Space; FD, fractal dimension [19]; and structural model index, SMI [20].

Peripheral quantitative computed tomography

The distal metaphysis, 1.4 mm proximal to the growth plate and mid-diaphysis of femurs (5 mm proximal to the midpoint), was scanned by a Research SA+ pQCT model (Norland Stratec, Berkenfeld, Germany) with a tube voltage of 50 kV and a tube current of 550 µA using a voxel size of

$80 \times 80 \times 46$ (μm). The cortical bone was defined as the area of bone mineral density (BMD) >690 mg/mm^3 , while a threshold of 395 mg/mm^3 at the contour mode 1 was set to define trabecular bone in the bone marrow. Total BMD (mg/cm^3) and the content, BMC (mg/mm), were presented as metaphyseal mineral properties. In addition, the cortical thickness (CTh), cross-sectional moment of inertia (CSMI), and polar stress/strain index (pSSI), an index of strength [21], were calculated.

Mechanical properties of femurs

The bone strength of the femoral diaphysis and distal epiphysis was evaluated using three-point breaking tests and compression tests using a MZ-500 s device (Maruto, Tokyo, Japan). The crosshead speed in the three-point breaking test and the compression test was 10 and 1.0 mm/min, respectively. In the latter, the distal epiphysis, approximately 3.0 mm thick, was compressed to 1.5 mm. The ultimate load (UL) and stiffness (s) were determined from the load–displacement curve and were converted to the material properties. Ultimate stress (US) was calculated by using the equation $US = (UL \times d \times L) / (8 \times \text{CSMI})$, where d is the diameter at midshaft, and L is the support span at the bottom (10 mm). The elastic modulus, E , was calculated by using the equation $E = (s \times L^3) / (48 \times \text{CSMI})$.

Confocal Raman spectroscopic measurements

Confocal laser Raman microspectroscopy was used to examine the composition and relative amounts of the

mineral and matrix produced in the tibia. Raman spectroscopy is particularly useful for bone analysis because it probes the molecular and ionic vibrations of the mineral and matrix component in unprocessed preparations that preserve these components. The innate, prominent vibrations were measured as described by Tarnowski et al. [22]. Crystallinity was determined using the method reported by Yerramshetty et al. [23] as the inverse of the width of the phosphate symmetric stretch band ($\text{PO}_4^{3-} \nu_1$ at 959 cm^{-1}) at half the maximum intensity value. A Nicolet Almega XR Dispersive Raman microscope system equipped with the OMNIC Atlus™ imaging software program (Thermo Fisher Scientific, MA, USA), which enable to map a small area less than $1 \mu\text{m}^3$ on the bony microsurface of the cortical bone on the video microscope stage control. A high brightness, low-intensity laser operating at 780 nm was used as the excitation source with a laser power of 35 mW. Each spectrum is the sum of ten 10-s measurements. The spectral resolution of the Almega XR under the conditions used was 3.85 cm^{-1} . For each femur, one averaged Raman image was acquired in the middle of the anterior cortical bone by the ten 10-s measurements.

Statistical analysis

All data values were expressed as the means \pm standard deviation (SD). Unless otherwise mentioned, the group means for each parameter were determined for the 8-week midpoint experimental results and compared using a one-way analysis of variance (ANOVA), with the post hoc Tukey–Kramer test.

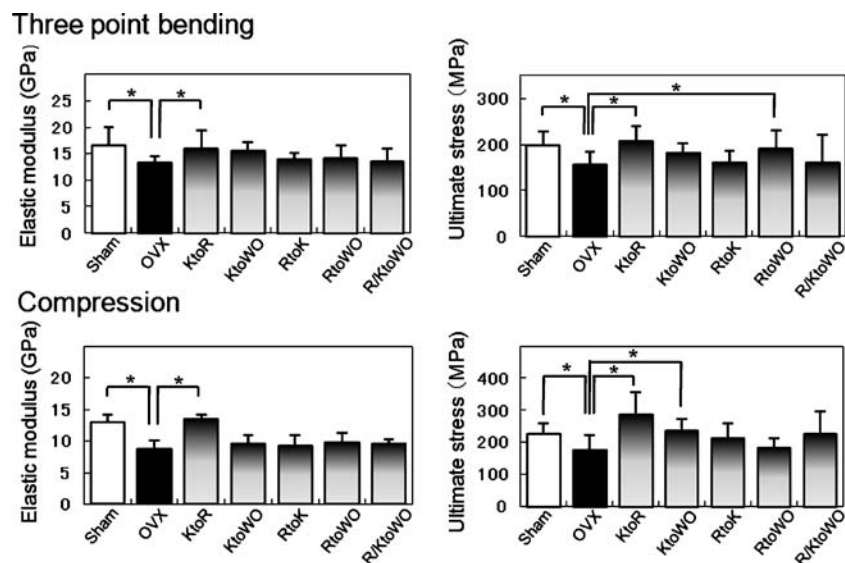


Fig. 1 Material properties of the femoral mid-diaphysis (*top panels*) and of the femoral distal epiphysis (*bottom panels*). After the 16-week treatments with risedronate and/or MK-4, the three-point bending test and compression test were carried out as described in the “Materials and methods” section for the diaphyseal and epiphyseal mechanical strength

analyses, respectively. *Open and filled bars* represent the sham and OVX controls, respectively. *The bars of graded shading* represent the treatment groups. The data are expressed as the means \pm SD and compared using an ANOVA and post hoc Dunnett’s multiple comparison test vs. OVX controls. $*p < 0.05$ was considered significant

Dunnett's multiple comparisons test was used for 16-week treatment groups with the OVX group as a reference. The probability values of $p < 0.05$ were considered to be statistically significant for all the comparisons. The Stat View software package (Stat View 5.0; Abacus Concepts, Berkeley, CA, USA) was used for all analyses.

Results

Body weight and length of femur

The body weight, which was 33.6 ± 2.1 at the ovariectomy (–4 weeks), ranged from 37.4 ± 2.1 to 40.3 ± 3.0 g at 0 week in the sham and OVX groups. At 8 and 16 weeks,

the range in all groups was between 40.9 ± 2.7 and 44.3 ± 4.3 g and 43.6 ± 7.5 and 49.4 ± 7.0 g, respectively. The length of the femur at the time they were killed ranged between 17.5 ± 0.6 and 17.8 ± 0.4 mm. Neither body weight nor the length of femur showed any significant difference in any of the treatment groups compared to the OVX or sham group (data not shown). While the body weight in OVX groups tended to be larger at 0 and 8 weeks, no significant effect was detected (data not shown). No intergroup difference was detected either (data not shown).

Mechanical tests of femurs after the 16-week treatments

As shown in the Fig. 1, the bending strength of the femoral diaphysis (top panels) and the compressive strength of the

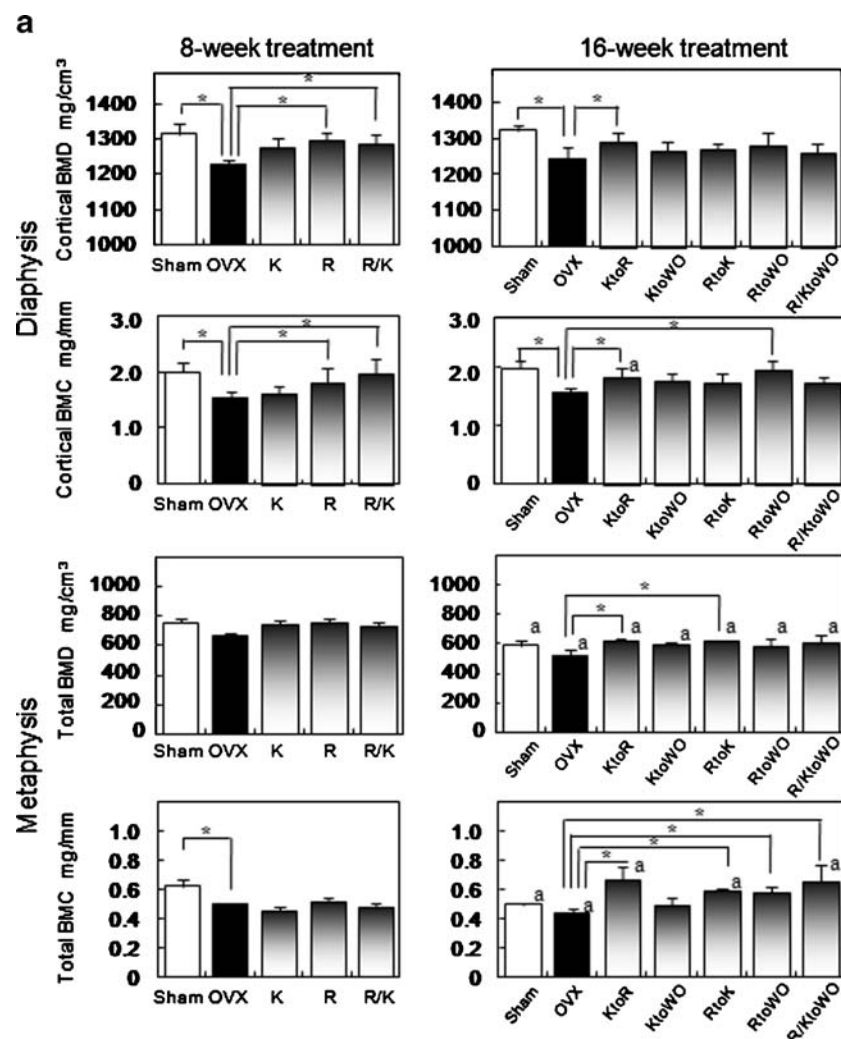


Fig. 2 Mineral and geometric properties at 8-week midpoint and 16-week termination. **a** Bone mineral density (BMD) and content (BMC) in femur diaphysis and metaphysis and **(b)** cortical thickness, CSMI, and the polar SSI in femur diaphysis. The data are expressed as the means \pm SD, and $*p < 0.05$ represents significance. ANOVA followed by post hoc Tukey–Kramer paired multiple comparison test (at

8 weeks) or Dunnett's multiple comparison test vs. OVX controls (at 16 weeks) were used. At 16 weeks, significance ($p < 0.05$) of each parameter determined by t test against the corresponding 8-week midpoint value was marked by *a*. The bars are similarly marked to those in Fig. 1

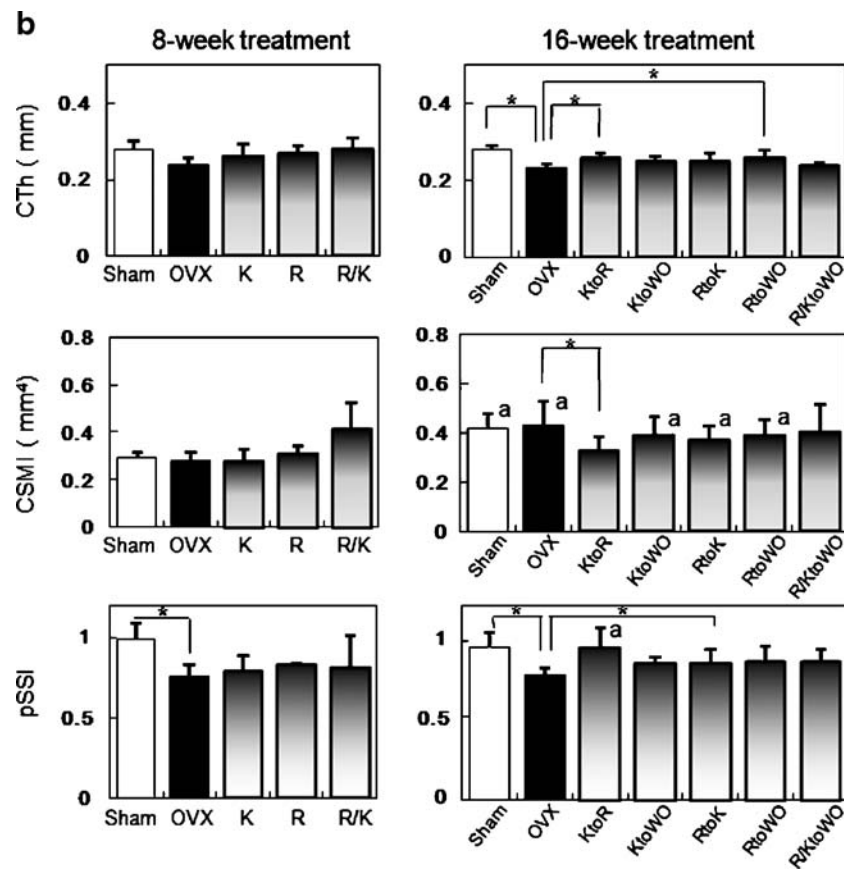


Fig. 2 (continued)

femoral distal metaphysis (bottom panels) were tested. In comparison to the OVX bone, a significant difference was detected in the sham bone as revealed by the elastic modulus as well as the ultimate stress values. The deficit in the OVX was restored in K to R in all four parameters, R to WO in the ultimate stress by three-point bending test, and K to WO in the ultimate stress by compression test.

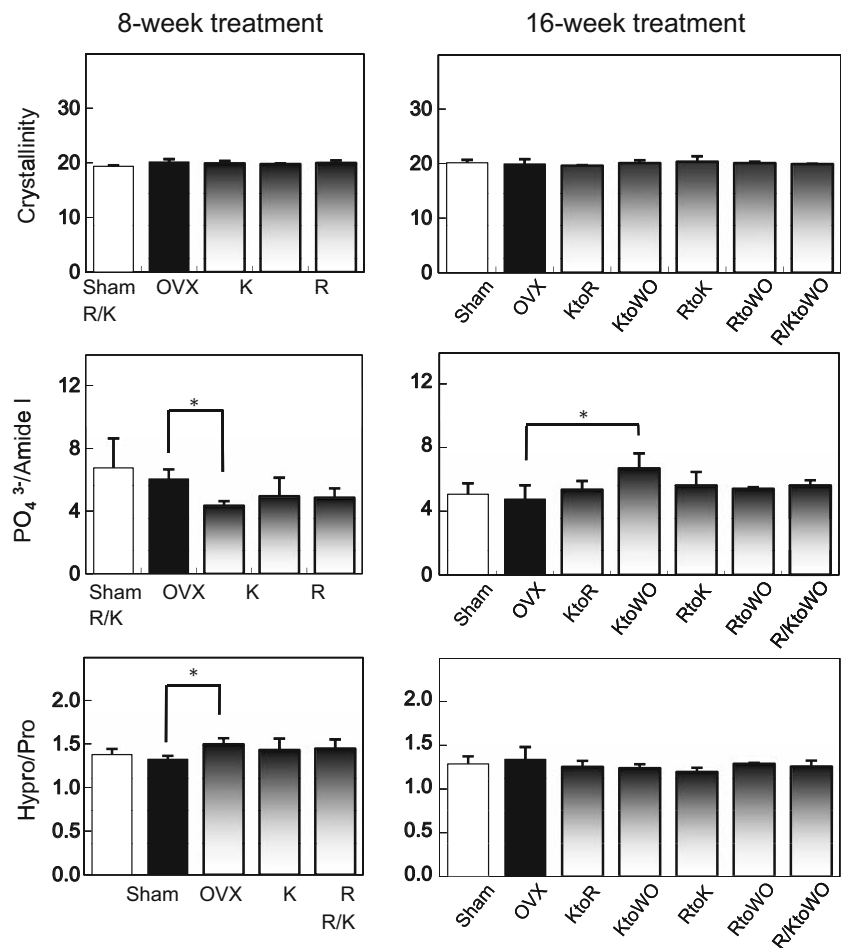
Changes in the cortical bone quality

Right panels in Fig. 2 show the results at the 16-week termination. The OVX control group showed a significant decrease in the cortical BMD and BMC as well as thinning of the cortical thickness and a decreased pSSI in comparison to the sham group. The final 16-week cortical BMD, BMC (Fig. 2a) and thickness (Fig. 2b) did not significantly change by any treatment from the 8-week stage except in the K to R cortical BMC. Among the treated groups, only the K to R group showed significantly higher values (lower in CSMI) than the OVX controls in all the parameters presented. Unless followed by risedronate, treatment by MK-4 did not significantly increase mineral content or density neither in diaphysis nor in metaphysis. Only in the K to R group was

CSMI significantly smaller than the 16-week OVX control. The K to R femur alone also raised the pSSI value, the calculated index of strength, to the levels of the sham group during the later 8-week treatment by risedronate (Fig. 2b). When we compare CSMI values in the 16-week treatment groups to their respective 8-week values by the Student's *t* test, many groups, including sham, OVX, R to K, R to WO, and K to WO, significantly increased the values during the later 8-week treatment. In the R/K to WO, CSMI retained similar high values with similarly large SD to the OVX-R/K 8-week midpoint. The R to WO group but not R/K to WO was also distinct showing significantly higher values than the OVX control in both cortical BMC and thickness.

Analysis of Raman spectra (Fig. 3) revealed that the resolvable mineral factor was of a carbonated apatite almost identical to what was reported by Tarnowski et al. [22] ($\text{PO}_4^{3-\nu_1}$, 959 cm^{-1} ; $\text{PO}_4^{3-\nu_4}$, 580 cm^{-1} ; $\text{CO}_3^{2-\nu_1}$, 1,072 cm^{-1}), and the matrix factor was of a collagenous protein (amide I, 1,666 cm^{-1} ; amide III, 1,242 and 1,269 cm^{-1} ; CH_2 wag, 1,450 cm^{-1} ; hydroxyproline, 855 and 878 cm^{-1} ; proline, 919 cm^{-1} ; HPO_4^{2-} , 1,005 cm^{-1} ; data not shown). While mineral properties such as the crystallinity were unchanged in all groups throughout the

Fig. 3 Analysis of femur diaphyseal cortex by confocal laser Raman microspectroscopy. $\text{PO}_4^{3-}\nu_1$ at 959 cm^{-1} was used as a mineral parameter and the amide I at $1,666\text{ cm}^{-1}$, and hydroxyproline (Hypro) at 855 and 878 cm^{-1} and proline (Pro) at 919 cm^{-1} were used as matrix parameters. The spectral band intensity by peak area, height for the Hypro/Pro ratio, or the band width for crystallinity was collected at each band as described in the “Materials and methods” section. The values are compared among 8- and 16-week samples, respectively, and between 8- and 16-week samples as in Fig. 2. Except for the Hypro/Pro ratio, which was based on the Fischer's LSD test, statistical analysis used was the same as in Fig. 2



16-week experiment, the cortical mineral to matrix ratio measured by $\text{PO}_4^{3-}\nu_1/\text{amide I}$ was significantly lower, and Hypro/Pro ratio was significantly higher only in OVX-K at 8 weeks than the OVX controls. At 16 weeks, the $\text{PO}_4^{3-}\nu_1/\text{amide I}$ ratio significantly increased in K to WO alone, revealing the decreased collagenous matrix by the MK-4 withdrawal. Hypro/Pro ratio was all similar at 16 weeks.

Changes in the trabecular architecture

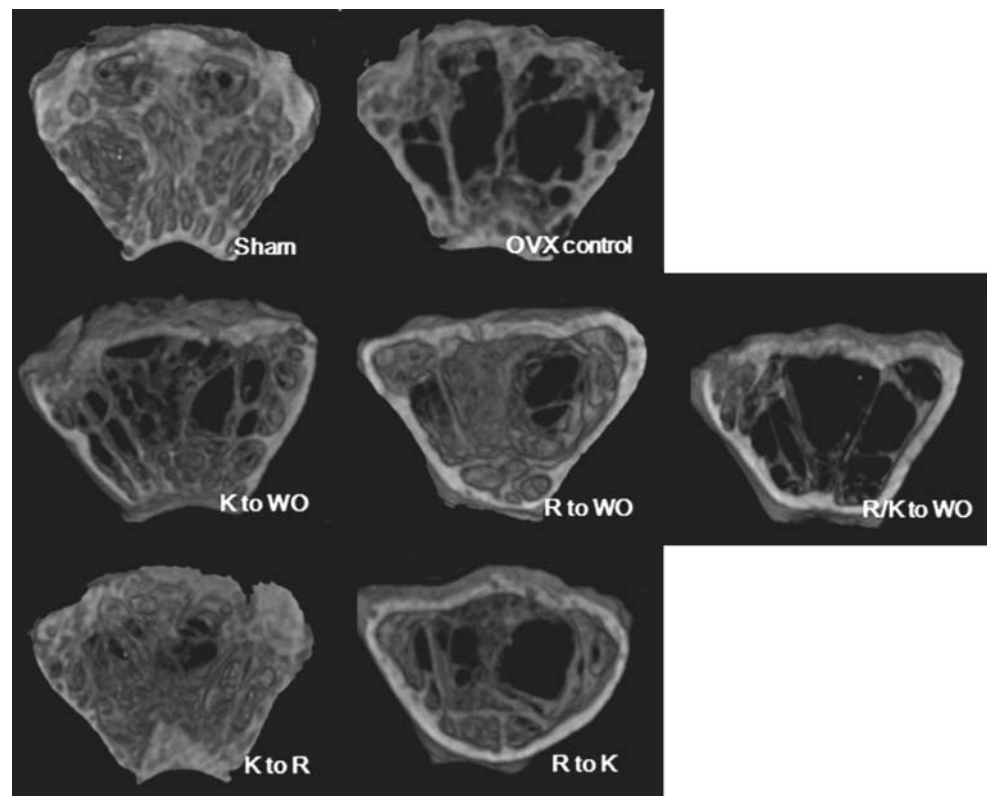
The effects of K to R on the distal metaphyseal (Fig. 2a) and the distal epiphyseal trabeculi (Table 2 and Fig. 4) were also quite significant. In Tables 1 and 2, the structural parameters by micro-CT analysis are summarized. In comparison to the OVX controls, sham group showed significant differences in the BV, BS, BV/TV, Tb.Th, Tb.N, and FD (larger) and Tb.Sp (smaller) at 8 weeks. All three 8-week treatment groups, OVX-R, K, and R/K, showed significant difference from the OVX group in many parameters (Table 1). Of note, the concomitant administration, OVX-R/K, was no more effective than the OVX-K or OVX-R monotherapy. The effect of 16-week treatment with MK-4 and/or risedronate was as follows. Both K to R and K

to WO groups showed significantly better BV, BS, BV/TV, Tb.N, and Tb.Sp values in comparison to the OVX group ($p < 0.01$ in K to R). Figure 2a also shows that K to R and R to K groups were higher in the metaphyseal total BMD and BMC, while BMC values were also higher in the R to WO and R/K to WO. Risedronate raised metaphyseal total BMC by more than 50% in K to R during the later 8 weeks. On the other hand, the R to WO and R/K to WO groups significantly lowered Tb.Th in comparison to the OVX control group (Table 2). In contrast to the MK-4 withdrawal, R to WO and R/K to WO lowered other trabecular structural parameters as well to the levels of OVX controls (Table 2). Figure 4 represents reconstructed 3-D images at 16 weeks of the distal epiphyseal region. The trabecular architecture looked poor in the OVX control and R/K to WO groups.

Discussion

Generally, drugs targeting different functions are combined for multidrug therapy with the expectation of complementary action. For vitamin K, however, even the efficacy by itself is still controversial. Earlier, low concentrations of

Fig. 4 Representative 3-D images of the distal epiphysis between 1.5 and 2.75 mm proximal to the growth plate after the 16-week treatments. Micro-CT images were reconstructed as described in the “Materials and methods” section



circulating vitamin K have been associated with bone fractures [24] and with low bone mineral density [25]. The undercarboxylated osteocalcin was associated with fracture risk [26, 27], and its reduction by the vitamin K intake was reported without the effect on BMD [28]. Furthermore, a vitamin K intake level of less than 109 $\mu\text{g}/\text{day}$ has been reported to be a risk factor for hip fracture in women [29]. Shiraki et al. treated postmenopausal patients with 45 mg/day MK-4 and reduced the new fractures to one third. Their lumbar BMD was found to be significantly higher than that observed in the control women [10]. In a more recent study, the combination of alendronate with 45 mg/day MK-4 was reported to be superior to alendronate monotherapy in

decreasing undercarboxylated osteocalcin, increasing femoral neck BMD and decreasing the urinary deoxypyridinoline [30]. In the animal studies, a much higher dosage of 30–50 mg MK-4/kg/day has been used, thus resulting in a significantly higher mineral content in cortical bone without bisphosphonate [31]. However, the results are inconsistent among different animals or strains [16–18, 32–34]. In the present study, we did not observe significant increase in BMD or BMC at the lower level of $\sim 100 \mu\text{g}/\text{kg}/\text{day}$ unless MK-4 was followed by risedronate.

Vitamin K₂ has been known to be essential for the γ -carboxylation of osteocalcin [35]. Therefore, the function was once assumed through activating osteoblasts and

Table 1 Three-dimensional structural parameters of epiphyseal trabecular bone at 8 weeks

	BV (mm^3)	BS (mm^2)	BV/TV(%)	Tb.Th (μm)	Tb.N (/mm)	Tb.Sp (μm)	FD	SMI
Sham	0.69 \pm 0.15 ^a	24.7 \pm 5.3 ^a	30.5 \pm 5.8 ^b	54.7 \pm 3.2 ^b	5.5 \pm 0.9 ^b	137.3 \pm 75.1 ^a	2.3 \pm 0.0 ^b	2.7 \pm 0.2
OVX control	0.27 \pm 0.05	11.8 \pm 1.8	14.1 \pm 4.7	45.2 \pm 1.3	3.1 \pm 0.5	334.7 \pm 26.0	2.1 \pm 0.0	2.7 \pm 0.2
OVX-K	0.67 \pm 0.05 ^a	27.3 \pm 1.7 ^a	29.5 \pm 1.8 ^a	48.2 \pm 0.9	5.8 \pm 0.2 ^a	127.6 \pm 24.5 ^a	2.3 \pm 0.0 ^b	3.1 \pm 0.2
OVX-R	0.56 \pm 0.01 ^a	22.8 \pm 1.5 ^a	22.7 \pm 1.8	47.7 \pm 1.2	4.6 \pm 0.5	190.9 \pm 19.1 ^b	2.2 \pm 0.0	3.2 \pm 0.3 ^{b,c}
OVX-R/K	0.65 \pm 0.06 ^a	24.3 \pm 1.7 ^a	25.9 \pm 1.8 ^b	50.6 \pm 0.9	4.8 \pm 0.2	165.7 \pm 24.9 ^b	2.2 \pm 0.0	3.4 \pm 0.3 ^{b,c}

Data are expressed as means \pm SD. Group comparisons were performed by analysis of variance (ANOVA) followed by Tukey–Kramer test. No significant difference was detected between OVX groups

^a $p < 0.01$ vs OVX controls

^b $p < 0.05$ vs OVX controls

^c $p < 0.05$ vs sham

Table 2 Three-dimensional structural parameters of epiphyseal trabecular bone at 16 weeks

	BV (mm ³)	BS (mm ²)	BV/TV (%)	Tb.Th (μm)	Tb.N (/mm)	Tb.Sp (μm)	FD	SMI
Sham	0.14±0.05 ^b	8.0±3.2 ^b	6.3±2.0 ^b	35.4±2.0	1.8±0.6 ^b	602±273 ^b	1.9±0.0	2.6±0.2
Control	0.08±0.03	5.1±1.6	3.6±1.0	32.0±3.1	1.1±0.3	944±279	1.8±0.1	2.7±0.1
K to R	0.22±0.06 ^a	12.9±2.7 ^a	8.9±2.4 ^a	34.2±3.9	2.6±0.5 ^a	369±100 ^a	2.0±0.1	2.5±0.1
K to WO	0.15±0.06 ^b	9.8±3.8 ^b	6.7±2.6 ^b	31.0±3.8	2.1±0.8 ^b	536±291 ^b	1.9±0.1	2.5±0.1
R to K	0.14±0.03 ^b	7.8±1.9	6.0±1.4	33.6±3.5	1.7±0.4	733±376	1.9±0.1	2.6±0.1
R to WO	0.07±0.03	5.6±2.3	3.5±1.0	26.0±1.8 ^a	1.3±0.3	771±225	1.7±0.1	2.8±0.1
R/K to WO	0.10±0.04	6.8±2.7	3.9±1.7	27.7±2.3 ^b	1.4±0.6	828±397	1.8±0.1	2.8±0.1

Data are expressed as means±SD. Group comparisons were performed by analysis of variance (ANOVA) followed by Dunnett's test vs. OVX controls

^a $p < 0.01$

^b $p < 0.05$

leading them to enhanced mineralization [36]. The mice genetically deficient for osteocalcin, however, exhibited the gain in bone mass instead of loss [37], suggesting that the osteoprotective action of vitamin K is mediated by some other pathways. Recent reports showed that vitamin K₂ activates osteoblastic transcription of extracellular matrix-related genes [38] through steroid and xenobiotic receptor (SXR)/pregnane X receptor (PXR)-mediated Msx2 gene transcription in cooperation with the estrogen-bound ERα [14].

According to the findings of our 8-week administration, only the MK-4 monotherapy at the dietary level resulted in cortical bone matrix formation and maturation without significant increase in BMD or BMC. It was shown that vitamin K₂ not only stimulates cortical bone matrix formation but also accelerates proline hydroxylation, which is a prerequisite for collagen cross-linking to achieve a mature collagenous matrix. Whether the enzymes involved in these processes are the target of vitamin K₂ or not is yet to be resolved. In addition, MK-4 alone provided significant effect in most of the structural parameters of femoral trabecular bone. On the other hand, risedronate, at 0.25 mg/kg/day, was certainly effective, alone or in combination with MK-4, in femoral cortical BMD, BMC, and some trabecular structural parameters in the 8-week treatment. Of note, however, the 8-week concomitant administration was no more effective than each effective monotherapy. This led us to investigate the sequential administration of the two drugs with the same total dosage. The resulting final mechanical properties at 16 weeks were significantly better than the OVX controls only in K to R group. Despite of beneficial effects of 8-week MK-4 pretreatment on the cortical bone matrix, enhanced degradation of collagen seems to occur if the MK-4 is discontinued. In other words, the elevated 16-week mineral/matrix ratio in K to WO is distinct from the lowest 8-week midpoint ratio in the OVX-K. In contrast, the K to R

group retained a much lower mineral/matrix ratio at 16 weeks. Since the K to WO mineral value, the numerator, is lower than the K to R value judged from the cortical BMD, the higher mineral/matrix ratio in K to WO was derived from the denominator, the smaller matrix value. It suggests either the collagen degradation or the decreased synthesis after the MK-4 withdrawal. An elevated serum CTx value was observed in K to WO in the later 8 weeks (data not shown). During the later 8-week treatment in K to R, risedronate clearly prevented the increase in CSML, which occurred in K to WO. The lack of such prevention as well as the lack of other beneficial effects found in K to R cortex, such as the higher/larger BMD, BMC, and thickness, would explain why no significant effect was detectable in K to WO by the three-point bending test. In the MK-4 treated pre-OVX rats, Iwamoto et al. reported the elevated eroded surface as well as the bone formation rate that remained high after the MK-4 withdrawal [16]. The cellular mechanisms of the elevated collagen degradation therefore have to be confirmed in the future.

In the compression test, the ultimate stress parameter of K to WO as well as of K to R was significantly larger than the OVX control. This result was supported by the significantly better parameters of the trabecular structure in K to WO such as BV, BS, BV/TV, Tb.N, and Tb.Sp in comparison to the OVX controls. No such benefit was observed in R to WO and R/K to WO. The difference in the effect of MK-4 withdrawal on cortical bone and trabecular bone may be related to the distinct distribution of ERα vs. ERβ [39] or the different ERα signaling pathways [40], on the assumption that vitamin K and estrogen via the ERα cooperatively promote the osteoblast function through the Msx2 gene induction [14].

Concomitant administration of risedronate and MK-4 is probably not recommended because R/K to WO was generally not beneficial except in the metaphyseal total BMC. In addition, R to WO but not R/K to WO cortical

thickness and BMC are significantly higher at 16 weeks than the OVX control, resulting in the increased ultimate stress only in R to WO. Since OVX-R and OVX-RK at 8 weeks exhibited similar cortical thickness and BMC values, the negative effect of RK withdrawal is apparent. The continuous 16-week administration of risedronate and MK-4 (R/K to R/K) was not beneficial in any parameters tested, including the metaphyseal total BMC (data not shown). Although R to K also showed a significantly positive effect in metaphyseal total BMD and BMC, it is probably not recommended to follow R by K because none of the benefits available in the cortex of R to WO was seen in R to K. In conclusion, prior 8-week treatment with MK-4 followed by the 8-week risedronate prevented the shortcomings of individual drugs and significantly increased femur strength of ICR mice compared to the OVX controls.

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Conflicts of interest None.

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