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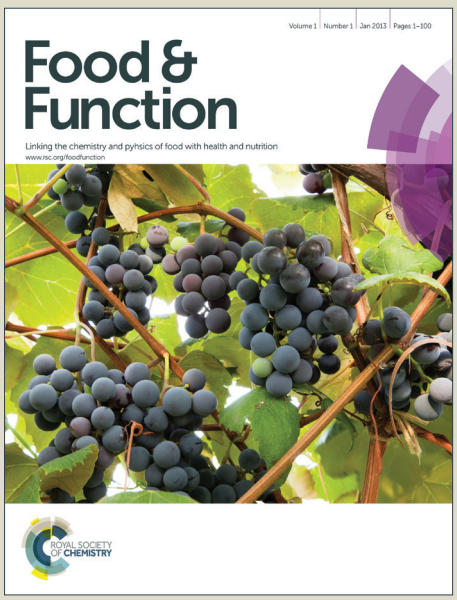

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The inhibitory effect of vitamin K on RANKL-induced osteoclast differentiation and bone resorption

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Running title: vitamin K inhibits osteoclast differentiation and resorption

Abstract

To further understand the correlation between vitamin K and bone metabolism, the effects of vitamins K_1, menaquinone-4 (MK-4), and menaquinone-7 (MK-7) on RANKL-induced osteoclast differentiation and bone resorption were comparatively investigated. Vitamin K_2 groups (MK-4 and MK-7) were found to significantly inhibit RANKL-medicated osteoclast cell formation of bone marrow macrophages (BMMs) in a dose-dependent manner, without any evidence of cytotoxicity. The mRNA expression of specific osteoclast differentiation markers, such as c-Fos, NFATc1, OSCAR, and TRAP, as well as NFATc1 protein expression and TRAP activity in RANKL-treated BMMs were inhibited by vitamin K_2, although MK-4 exhibited a significantly greater efficiency compared to MK-7. In contrast, the same dose of vitamin K_1 had no inhibitory effect on RANKL-induced osteoclast cell formation, but increased the expression of major osteoclastogenic genes. Interestingly, vitamin K_1, MK-4 and MK-7 all strongly inhibited osteoclastic bone resorption ($p<0.01$) in a dose dependent manner. These results suggest that vitamin K_1, MK-4 and MK-7 have anti-osteoporotic properties, while their regulation manners on osteoclastogenesis are somewhat different.

Keywords: bone marrow macrophages, bone resorption, osteoclast, RANKL, vitamin K

1. Introduction

Bone is a highly dynamic organ that maintains its homeostasis through a delicate balance between osteoclastic bone resorption and osteoblastic bone formation.[1] An imbalance between osteoclast and osteoblast activity is associated with diseases including rheumatoid arthritis and osteoporosis, which result from excessive osteoclast activity.[2,3] Osteoclasts are large multinucleated cells that originate from the hematopoietic stem cell macrophage lineage under the regulation of two main cytokines expressed in osteoblasts: receptor activator of macrophage colony stimulating factor (M-CSF) and nuclear factor-κB ligand (RANKL).[4] M-CSF is a secreted cytokine that provides survival signals to osteoclasts, while RANKL is a key cytokine that regulates osteoclastogenesis and bone resorption through binding to its receptor RANK.[4,5]

There is accumulating evidence indicating that vitamin K is involved in bone metabolism and may have beneficial effects on osteoporosis.[6,7] In nature, there are two biologically active types of vitamin K: vitamin K_1 (phylloquinone) and vitamin K_2 (menaquinone). Vitamin K_1 is a single compound widely distributed in plants, while vitamin K_2 is a series of vitamers with multi-isoprene units at the 3-position of the naphthoquinone, which is primarily of animal origin or from bacterial synthesis.[8] The commonly used nutritional supplement menaquinone-4 (MK-4) contains four isoprene units while menaquinone-7 (MK-7) contains seven.[9] These vitamin K species are well known for their essential role during γ-carboxylation of osteocalcin in osteoblasts, as noncarboxylated osteocalcin cannot bind to hydroxyapatite in mineralized tissues, suggesting that the primary mechanism underlying the

protective influence of vitamin K on bone may involve carboxylation of osteocalcin.[7,10] Regarding their effects on bone resorption, previous studies have reported that vitamin K_2 (MK-4) but not vitamin K_1 inhibited bone resorption by suppressing prostaglandin E_2 (PGE_2) synthesis in co-culture of spleen cells and stromal cells, and subsequently inhibited osteoclast formation.[11,12] In contrast, data from some other studies demonstrated that both vitamin K_1 and MK-4 inhibited osteoclast formation in an unfractionated bone marrow co-culture system.[13,14] These results are contradictory. The mechanisms for the anti-osteoclastogenic properties of these vitamin K species are poorly defined, although it has been suggested that MK-7 may have a direct suppressive effect on osteoclast differentiation and activity by down-regulation of NF-kB activation.[7] However, comparative studies about the effects of vitamin K_1, MK-4 and MK-7 on RANKL-induced osteoclast differentiation and bone resorption have not been adequately carried out.

In this study, the effects of vitamin K_1, MK-4 and MK-7 on RANKL-induced osteoclast differentiation, activation, and signaling pathways were comparatively investigated.

2. Materials and methods

2.1. Materials

Menaquinone-7 (MK-7, 97% purity) was highly purified from Korean traditional fermented soybean paste (*cheonggukjang*), which was made through fermentation of boiled soybeans at 43°C for 36 h by adding *Bacillus subtilis* KCTC 12392BP. MK-7 was extracted from *cheonggukjang* with 1.2-fold of 2-propanol and 2.4-fold of *n*-hexane, at room temperature for

1 h.[9] The organic layer was collected and concentrated under reduced pressure to yield the oil residue, then dissolved in ethanol and filtered through a 0.45-μm membrane filter. The ethanol solution was applied to a silica gel column (230-400 mesh, Merck, Dannstad, Germany) and eluted with a hexane-methanol solvent system (100:0, 75:25, 50:50, 25:75, 0:100; each 200 ml, v/v) to yield purified MK-7. Finally, the purity of isolated MK-7 was verified by UPLC/MS/MS (Waters, Milford, MA, USA), and dissolved in ethanol at a concentration of 10 mM as a stock for further application. Vitamin K_1, menaquinone-4 (MK-4), actin and NFATc1 antibodies were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant mouse soluble RANKL and M-CSF were obtained from Koma Biotech (Seoul, Korea). Penicillin/streptomycin and α-minimum essential medium (α-MEM) were purchased from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was obtained from HyClone (South Logan, Utah, USA). All other chemicals were of analytical grade or met the standards required for cell culture experiments.

2.2. Mouse bone marrow macrophage preparation and osteoclast differentiation

Bone marrow cells were isolated from 5-week-old male ICR mouse (Samtako, Osan, Korea) femurs and tibias according to previous studies,[4] with slight modifications. All experiments performed in this study were in accordance with the animal experiment guidelines of the Institute Committee of Chonbuk National University and Wonkwang University. Briefly, bone marrow cells were collected by flushing the femurs and tibias with PBS, and red blood cells were removed by culture with Gey's solution. Following centrifugation, cells were

suspended in α-MEM supplemented with 10% FBS, antibiotic mixture (100 U/ml penicillin G, 100 μg/ml streptomycin), and cultured for two days in the presence of M-CSF (10 ng/ml) in petri dishes. Then, non-adherent cells were collected and used as bone marrow macrophages (BMMs), which serve as osteoclast precursors. To generate osteoclasts from the BMMs culture system, BMMs were seeded at 12×10^4 cells/well in α-MEM/10% FBS containing M-CSF (30 ng/ml) and RANKL (50 ng/ml) for 4 days in a 24-well plate with or without vitamin K species. Osteoclast cells were identified by staining for the tartrate-resistant acid phosphatase (TRAP) activity according to the method of Park et al.[15] Briefly, the cells were fixed with 3.7% formalin for 10 min, permeabilized with a methanol: acetone (1:1, v/v) mixture for 1 min, and then stained with TRAP (Sigma, St. Louis, MO, USA). TRAP-positive giant multinucleated cells with more than three nuclei were identified as osteoclasts and counted. TRAP enzyme activity was assayed in 96-well plates using pNPP (Sigma, St. Louis, MO, USA) as the substrate according to a previous method.[16]

2.3. Cytotoxicity assay

BMMs were seeded in 96-well plates at a density of 1×10^4 cells/well and cultured overnight. Cells were treated with M-CSF (30 ng/ml), and various concentrations of vitamin K species. After 3 days of culturing, cell proliferation was measured by 3-(4,5-dimethylthiazol-2-yl) -2,5diphenyl tetrazolium bromide (MTT) assay.[17] 100 μl of 500 μg/ml MTT solution was added to each well. Then the plate was incubated at 37°C. After 4 h, the medium was removed and 100 μl of DMSO was added to each well and mixed thoroughly to dissolve the

formazan crystals. The optical density was then measured at 490 nm using an ELISA reader (BMG Labtech, Offenburg, Germany).

2.4. Real time RT-PCR analysis

Total RNA was isolated with RiboEx reagent (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. The quantity and quality of the RNA were measured spectrophotometrically (Biospec-nano, Shimadzu biotech, Kyoto, Japan). Then single-stranded cDNA was synthesized from 1 μg of the total RNA by using a first-strand cDNA synthesis kit (TaKaRa, Tokyo, Japan). The cDNA was amplified by using the primer sets as following: c-Fos, 5'-CTG GTG CAG CCC ACT CTG GTC-3' (forward) and 5'-CTT TCA GCA GAT TGG CAA TCT C-3' (reverse); NFATc1, 5'-CTC GAA AGA CAG CAC TGG AGC AT-3' (forward) and 5'-CGG CTG CCT TCC GTC TCA TAG-3' (reverse); TRAP, 5'-CTG GAG TGC ACG ATG CCA GCG ACA-3' (forward) and 5'-TCC GTG CTC GGC GAT GGA CCA GA-3' (reverse); OSCAR, 5'-CTG CTG GTA ACG GAT CAG CTC CCC AGA-3' (forward) and 5'-CCA AGG AGC CAG AAC CTT CGA AAC T-3' (reverse); and GAPDH, 5'-ACC ACA GTC CAT GCC ATC AC-3' (forward) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (reverse).[1] PCR was performed using the Rotor Gene (model RG-6000; Corbett Research, Sydney, Australia) and the SYBR Green qPCR kit (Enzynomics, DaeJeon, Korea) in triplicates according to the manufacturer's instructions. Relative levels of c-Fos, NFATc1, TRAP, and OSCAR were normalized to GAPDH, and presented as the mean fold change as compared to controls.

140 **2.5. Western blot analysis**

141 BMMs or osteoclasts were lysed with RIPA buffer (Sigma, St. Louis, MO, USA) and
142 removed by centrifugation at 14,000 rpm for 15 min. The protein concentration was
143 measured with a Bicinconinic acid (BCA) kit (Pierce; Rockford, IL) and an equal amount of
144 each lysate (20 μg) was separated on an SDS-polyacrylamide gel. After separation, the
145 proteins were transferred to the PVDF membrane (Millipore, Billerica, MA) and blocked
146 with 5% skim milk for 2 h, then incubated with primary antibodies. Membranes were
147 incubated with the appropriate secondary antibodies coupled to horseradish peroxidase,
148 followed by an application of enhanced chemiluminescence reagents. Densitometric values
149 were quantified for each band with the Image J program version 2.0.

150

151 **2.6. Intracellular Ca^{2+} measurement**

152 Cultured BMMs were seeded on cover glass and RANKL (50 ng/ml) was treated with or
153 without vitamin K species as indicated. After 24 h of culturing, cells were loaded with 5 μM
154 fura-2/AM for 50 min at room temperature. Cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) was measured using
155 wavelengths of 340 and 380 nm, the emitted light was passed through a 510-nm cut-off filter
156 and was collected with a charge-coupled device camera. The images were digitized and
157 analyzed with a Meta Fluor system (Universal Imaging Co., Downingtown, PA, USA).

158

159 **2.7. Resorption assay**

160 For bone-resorption activity assay, BMMs (12×10^4 cells/well) were seeded on an

Osteo-Assay Plate (24 well, Corning, NY, USA) treated with RANKL (50 ng/ml) and M-CSF (30 ng/ml). The cells were incubated at 37°C in the presence or absence of vitamin K species for 10 days. The resorption area was determined by Image J software.

2.8. Statistical analysis

Data were analyzed using IBM SPSS statistical software (SPSS 19.0, IBM Corp., Armonk, NY, USA), and a significant difference between groups was determined by Duncan's multiple test at the level of $p<0.05$.

3. Results

3.1. Effect of vitamin K on RANKL-induced osteoclast differentiation

To determine the effects of vitamin K species on osteoclastogenesis, osteoclast cells were induced from mouse BMMs by addition of RANKL (50 ng/ml). As expected, the BMMs from the control group were successfully differentiated into mature TRAP-positive multinucleated osteoclasts, and vitamin K_1 showed no inhibitory effect yet slightly increased osteoclast formation, while vitamin K_2 (MK-4 and MK-7) efficiently reduced the formation of TRAP-positive osteoclasts ($p<0.001$) in a dose-dependent manner (Fig. 1A). Compared to MK-7, a high dose of MK-4 (20 mM) exhibited a greater efficiency (85.3%) in the suppression of osteoclast differentiation (Fig. 1B).

3.2. Cytotoxic effect of vitamin K

Vitamin K_2 generated a highly negative effect on osteoclast differentiation. However, this inhibitory effect could have been due to the viability and/or reduced proliferation of the osteoclast precursor cells. To exclude this possibility, an MTT assay was carried out to examine the cytotoxic effects of vitamin K species on BMMs. According to Fig. 2, vitamin K_1 demonstrated no inhibition on BMM proliferation. In addition, MK-4 and MK-7 did not exert any cytotoxic effects at the same doses at which they effectively inhibited osteoclast differentiation (Fig. 2), suggesting that osteoclastogenesis suppression by vitamin K_2 was not due to toxic effects on BMMs.

3.3. Effect of vitamin K on c-Fos, NFATc1, TRAP, and OSCAR mRNA expression

Osteoclast differentiation is regulated by the expression of various genes, that in response to RANKL and RANK binding. The c-Fos and NFATc1 genes play an essential role in osteoclast differentiation, and NFATc1 regulates OSCAR and TRAP expression.[18,19] In this study, the effects of vitamin K species on the RANKL-induced regulation of c-Fos, NFATc1, OSCAR, and TRAP expression were assessed. BMMs were pretreated with vitamin K species, and further stimulated with RANKL for 48 h. Results revealed that c-Fos, NFATc1, OSCAR, and TRAP mRNA expressions were significantly increased in response to RANKL treatment, and this increase was reinforced by vitamin K_1 (Fig. 3). In contrast, vitamin K_2 (MK-4 and MK-7) significantly inhibited c-Fos, NFATc1, and TRAP expression ($p<0.05$). MK-4, in particular, showed an extremely high inhibitory effect ($p<0.01$) on all four mRNA expression levels (Fig. 3). These results suggest that vitamin K_2 may inhibit osteoclast formation through the suppression of RANKL-induced c-Fos and NFATc1 expression.

3.4. NFATc1 protein expression and TRAP activity

NFATc1, a master regulator of osteoclastogenesis, auto-amplifies and regulates the expression of osteoclast specific genes and was reported to be the key transcription factor for osteoclast differentiation.[1,4] Thus, the effects of vitamin K species on NFATc1 protein expression were further verified by Western blot assay in the present study. NFATc1 protein levels were increased in response to RANKL (154%), and this expression was slightly enhanced by vitamin K_1 (161%). While in the case of vitamin K_2, both MK-4 and MK-7 significantly inhibited NFATc1 protein expression to 118% and 126% of the RANKL negative group, respectively (Fig. 4). Additionally, MK-4 had a reverse effect on this phenomenon and exhibited a dramatic reduction of NFATc1 protein expression compared with the control. These results demonstrate that the inhibitory effects of vitamin K_2 on osteoclastogenesis involve the inhibition of a major transcription factor, NFATc1.

In order to analyze the effects of vitamin K species on TRAP activity during osteoclastogenesis in more detail, BMMs were pretreated with vitamin K species, and further stimulated with RANKL. Following a culture period of 4 days, TRAP activity was assessed. The results indicated that vitamin K_1 increased TRAP activity in a dose-dependent manner (Fig. 1C). In contrast, MK-4 strikingly reduced this TRAP activity when the dose was greater than 1 μM ($p<0.001$). In the case of MK-7, it also showed a significant inhibitory effect ($p<0.01$) on TRAP activity at high doses (>10 μM), although this effect is minor compared to that with MK-4. These findings are in accordance with the Real-Time PCR results (Fig. 3), where vitamin K_1 enhanced TRAP mRNA expression, while vitamin K_2 inhibited its expression.

3.5. Effect of vitamin K on $[Ca^{2+}]_i$ oscillation

To confirm whether vitamin K species were involved in the induction of RANKL-induced Ca^{2+} oscillations, the effect of vitamin K species on Ca^{2+} responses in the presence of RANKL was investigated. Results indicate that that Vitamin K_1 treatment (Fig. 5B) slightly enhanced the frequency of the Ca^{2+} oscillations compared to the control group (Fig. 5A). On the other hand, vitamin K_2 treatment (MK-4 and MK-7) significantly reduced the frequency of Ca^{2+} oscillations induced by RANKL (Fig. 5CD). These results suggest that vitamin K species somehow regulate RANKL-induced Ca^{2+} oscillations.

3.6. Bone-resorbing activity

The effects of vitamin K species on the bone-resorption activity of osteoclasts were also investigated by using an osteo assay plate. BMMs were treated with RANKL (100 ng/ml) and cultured in the presence or absence of serial concentrations of vitamin K species on an osteo assay plate. After 10 days, numerous resorption pits had been generated by mature osteoclasts. The addition of vitamin K_1 (55%), MK-4 (70%) and MK-7 (55%) all strikingly inhibited the bone resorptive capacity of mature osteoclasts (p<0.01) at dose of 20 μM (Fig. 6).

4. Discussion

Accumulating evidence indicates that vitamin K_1, MK-4, and MK-7 are involved in bone metabolism, and have preventative effects against osteoporosis.[6,13,20-22] However, the direct effect of vitamin K species on osteoclast differentiation and the status of vitamin K during bone resorption are not fully understood yet. In the present study, the regulation effects of

vitamin K_1, MK-4 and MK-7 on osteoclastogenesis and bone resorption were compared.

In this study, vitamin K_1 slightly enhanced osteoclast cells differentiation by increasing expression of the major osteoclastogenic genes such as c-Fos, NFATc1, OSCAR, and TRAP (Fig. 3), as well as increased the protein expression of NFATc1 (Fig. 4) and TRAP activity (Figs. 1C). It has been well-established that RANKL stimulates osteoclast formation by activating c-Fos, which binds to the promoter region and activates NFATc1 expression; thereafter, c-Fos and NFATc1 act synergistically to up-regulate the expression of specific osteoclast-regulating genes, including OSCAR and TRAP.[23] Moreover, OSCAR acts as a costimulatory receptor for osteoclast generation by activating NFATc1 expression in turn, through costimulatory signaling mediated by FcRγ-associated immune-receptors.[24] Where, NFATc1 as the master transcription factor for osteoclastogenesis, has been reported auto-amplify its own gene expression by translocating into the nucleus after dephosphorylation by calcineurin and binding to its own promoter.[24] Results from this study consistently indicated that vitamin K_1 may play as a synergy factor during the process of osteoclasts formation induced by RANKL. In contrast to vitamin K_1, vitamin K_2 (MK-4 and MK-7) significantly suppressed RANKL-induced osteoclast formation, osteoclastogenic gene markers' expressions, protein NFATc1 expression, and TRAP activity in a dose-dependent manner without any evidence of cytotoxicity (Figs. 1-4). NFATc1 is regard as the master molecule to govern the late-stage of osteoclast differentiation. Notably, the activity of NFATc1 are known to be regulated by Ca^{2+} signaling relied on the tyrosine phosphorylation pathways.[4,25] In this study, vitamin K_1 treatment slightly enhanced the RANKL-induced Ca^{2+}

oscillation, whereas MK-4 and MK-7 reduced the frequency of Ca^{2+} oscillations in BMMs (Fig. 5). During osteoclastogenesis, the increased Ca^{2+} level mediates NFATc1 dephosphorylation and translocation into the nucleus.[4] Thus, the expression of NFATc1 (Fig. 4) regulated by vitamin K species is in accordance with the Ca^{2+} oscillation results. These findings demonstrate that the regulation effects of vitamin K species on osteoclast differentiation are associated with the Ca^{2+} oscillation.

Although the structural differences among vitamin K_1, MK-4 and MK-7 exist only in the side chain, the effects of these vitamin K species on osteoclast cell formation were different: vitamin K_2 (MK-4 and MK-7) inhibited osteoclast cell formation, whereas vitamin K_1 did not have this inhibitory effect. Similarly, a previous study reported that vitamin K_2 (MK-7) had a direct suppressive effect on RANKL-induced osteoclast-like cell formation, but the same dose of vitamin K_1 had no effect on osteoclast-like cell formation.[7] Moreover, Hara et al. reported that MK-4, but not vitamin K_1, inhibited osteoclast-like multinucleated cell formation in co-culture of spleen cells and stromal cells.[11] Collectively, these results suggest that the side chain of vitamin K may play an important role in the inhibitory effect of vitamin K_2 on RANKL-induced osteoclast differentiation, as well as in the spleen cell and stromal cell co-culture system. Moreover, the side chains of MK-4 and MK-7 share the same isoprene unit, which may be associated with their inhibitory role in RANKL-induced osteoclast cell formation. MK-4 has a side chain with four isoprene units in length and exhibited an extremely high inhibitory effect, compared to MK-7 (Fig. 1), which contains seven isoprene units. In a previous study, the effects of multi-isoprenyl alcohols with two to seven isoprene

293 units on TRAP activity induced by $1,25(OH)_2D_3$ were investigated in a spleen cell and
294 stromal cell co-culture system. The results indicated that only geranylegeraniol (side chain of
295 MK-4) with four isoprene units exerted a marked inhibitory effect.[11] However, in a more
296 recent investigation, the inhibitory effect of MK-4 and of its side chain component,
297 geranylegeraniol, on osteoclast formation has been reported in different manners.[12] Therefore,
298 both the side chain and the naphthoquinone ring of vitamin K may be associated with its
299 inhibitory effect on osteoclast cells formation, although the side chain may play a more
300 important role.

301 The effects of vitamin K species on osteoclastogenesis were not only limited to the
302 suppression of osteoclast cell differentiation; they also inhibit osteoclasts resorption. Vitamin
303 K_1, MK-4 and MK-7 all strikingly inhibited osteoclast resorption ($p<0.01$) in a
304 dose-dependent manner (Fig. 6). Our recent report demonstrated that ovariectomized rats
305 treated with MK-7 (8 μg/day) exhibited a significantly increase in trabecular number, bone
306 mineral content (BMC) and bone mineral density (BMD) ($p<0.01$).[6] Moreover, a previous *in*
307 *vivo* study also reported that vitamin K_1 has the beneficial effects on increasing both of the
308 femoral BMC and BMD, while MK-4 has the beneficial effects of increasing femoral BMC,
309 bone volume, width and bone strength parameters.[26] These findings suggest that vitamin K_1,
310 MK-4 and MK-7 have beneficial effects on the bone metabolism. According to their
311 molecular structure, the common part of vitamin K_1, MK-4 and MK-7 is limited to the
312 naphthoquinone ring. In addition, there is consistent evidence indicates that vitamin K_1 could
313 be converted into MK-4 in living body, extrahepatic tissue and osteoblastic cells by

exchanging of the side chain moiety.[27,28] Thus, the naphthoquinone ring of vitamin K is speculated to be important for its inhibition effect on bone resorption, although further investigation is needed to confirm this.

Interestingly, vitamin K_1 slightly enhanced osteoclast-like multinucleate cells formation (Fig. 1), but significantly inhibited osteoclast bone-resorption activity (Fig. 6), although the detail mechanism of action has not been established. It is believed that differentiation of mature osteoclasts need several steps, including fusion of the cells into osteoclast-like multinucleate cells, formation of the sealing zone and ruffled border, and active resorption.[29] During the bone resorption process, only fully matured osteoclast shows bone-resorptive activity. A previous study reported that TRAP-positive cells without osteoclast-maturing factors could not significantly form pits on bone slices, while addition of osteocalcin fragment induced the bone-resorbing ability.[29] Considering results from the present study, we can interpret that vitamin K_1 has no effects (or enhances) in early stage of osteoclasts formation, however, vitamin K_1 strongly inhibits osteoclast maturation or bone-resorptive function of matured osteoclasts.

5. Conclusion

In summary, vitamin K_2 (MK-4 and MK-7) has a direct inhibitory effect on RANKL-induced osteoclast differentiation, while vitamin K_1 showed a slight synergy effect on osteoclast formation. Vitamin K_2 inhibits osteoclast formation by dramatically suppressing specific osteoclast differentiation markers, including c-Fos, NFATc1 and TRAP expressions. Additionally, MK-4 with four isoprene units in its sidechain exhibited a significantly greater

efficiency to suppress osteoclastogenesis compared to MK-7, which contains seven isoprene units. However, after osteoclast formation, vitamin K_1, MK-4 and MK-7 all exhibit an inhibitory effect on bone-resorption activity. These results indicate that MK-7 from Korean traditional food *cheonggukjang* may be potentially useful as MK-4 for the prevention of osteoporosis.

Acknowledgements

This research was financially supported by the Ministry of Trade, Industry & Energy (MOTIE), Korea Institute for Advancement of Technology (KIAT) and Honam Institute for Regional Program Evaluation through the Leading Industry Development for Economic Region.

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Figure Captions

Fig. 1 Effect of vitamin K species on RANKL-induced osteoclast differentiation.

(A) Bone marrow macrophages (BMMs) were cultured for 4 days with M-CSF (30 ng/ml) and RANKL (50 ng/ml) in the presence of or absence of vitamin K. After 4 days, cells were fixed in 3.7% formalin, permeabilized in methanol: acetone (1:1) solution, and stained with TRAP solution. TRAP-positive cells were counted as osteoclasts. (B) Counting results of osteoclasts per well. (C) Effect of vitamin K species on RANKL-induced osteoclast TRAP activity. *p<0.05, **p<0.01, ***p<0.001 vs Control. Increased $^{\#\#}p$<0.01 vs Control.

Fig. 2 Effect of vitamin K on cell proliferation of bone marrow monocytes.

The cells were treated with vitamin K_1, MK-4, and MK-7 at different concentration (0.1 μM, 1.0 μM, 10 μM, and 20 μM, respectively) for 3 days, and cell viability was measured by MTT assay. Data were shown as mean ± SD of three independent experiments.

Fig. 3 Effect of vitamin K species on mRNA expression of c-Fos, NFATc1, TRAP, and OSCAR in BMMs treated with RANKL.

BMMs were cultured with or without vitamin K (20 μM) under RANKL stimulation (50 ng/ml) for 48 h. Expression of individual genes was normalized to GAPDH mRNA levels. Values are mean ± SD of triplicate reactions. Significant difference *p<0.05, **p<0.01 compared with Control. Increased $^{\#\#}p$<0.01, $^{\#\#\#}p$<0.001 compared with Control.

Fig. 4 Effect of vitamin K species on the protein expression of NFATc1.

421 BMMs were pretreated with or without vitamin K species (20 μM) and further stimulated with
422 RANKL (50 ng/ml) for 3 days. Actin was used as the internal control.

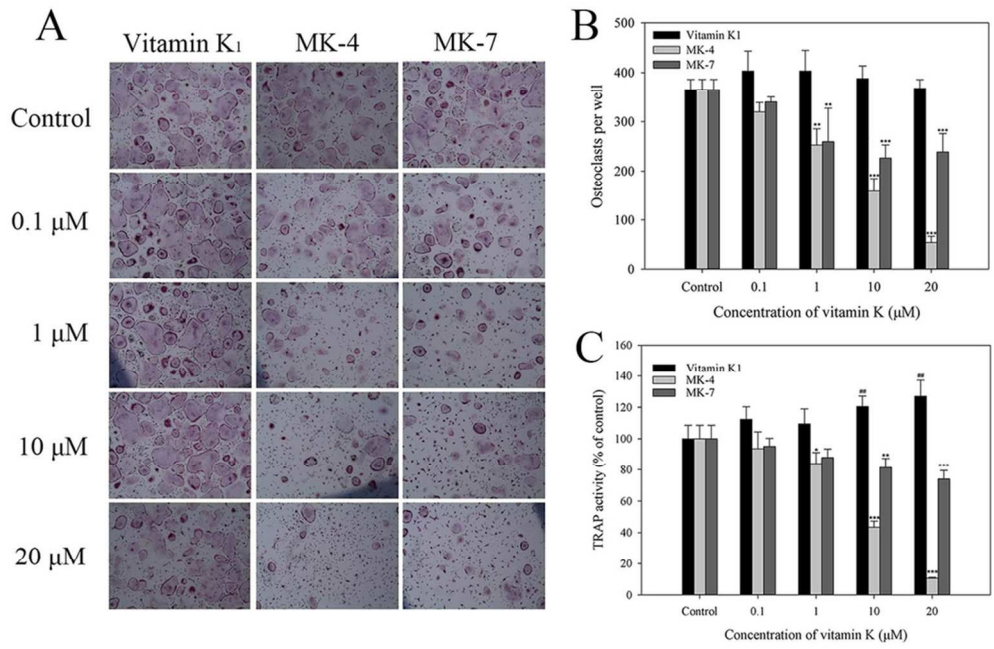
423

424 **Fig. 5** Effect of vitamin K species on RANKL-induced $[Ca^{2+}]_i$ oscillation.
425 BMMs were incubated with or without vitamin K (20 μM) in the presence of RANKL (50 ng/ml) for
426 24 h. (A) Control; (B) Treated with vitamin K_1; (C) Treated with MK-4; (D) Treated with MK-7.

427

428 **Fig. 6** Vitamin K species inhibit the bone resorption activity of osteoclasts.
429 BMMs were cultured with M-CSF (30 ng/ml) and RANKL (50 ng/ml) in the presence of or absence
430 of vitamin K species for 10 days. *p<0.05, **p<0.01 vs Control.

431



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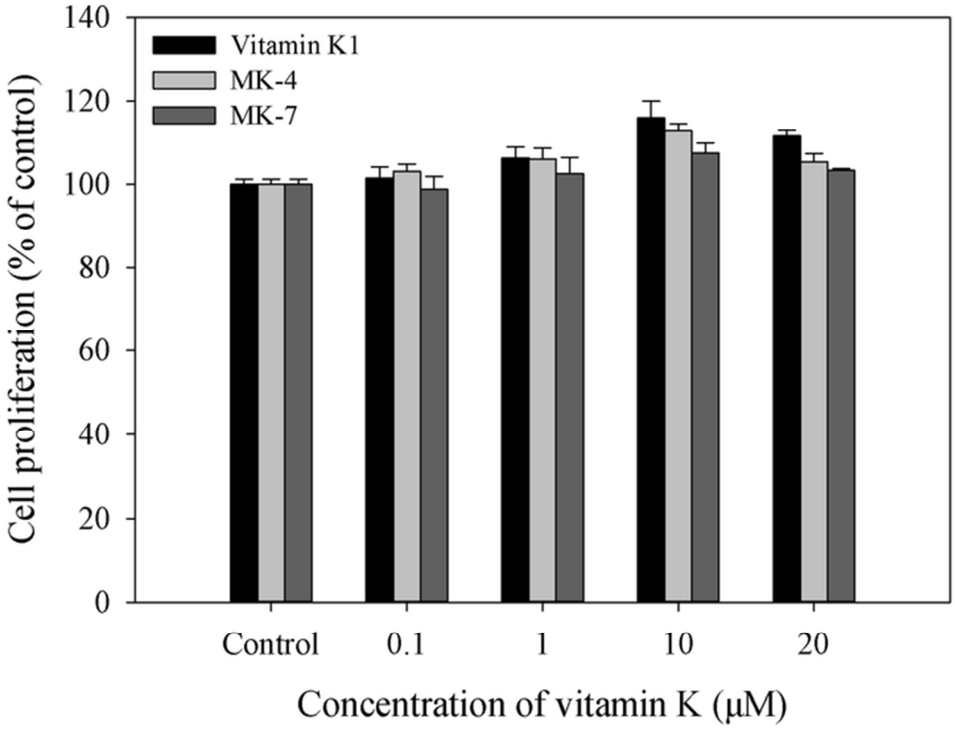

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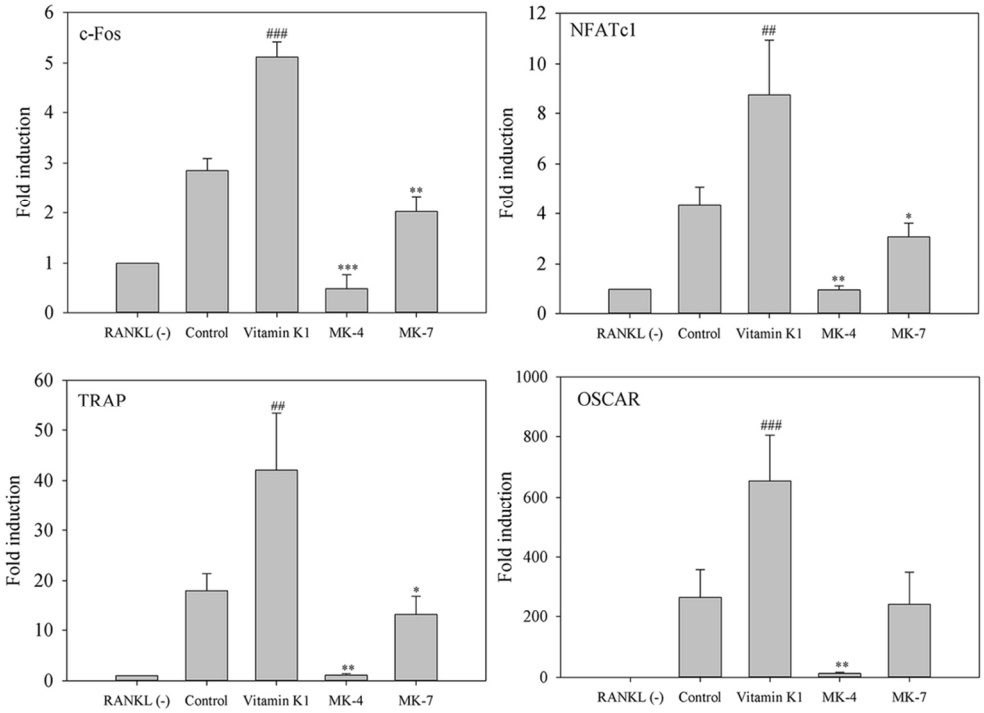

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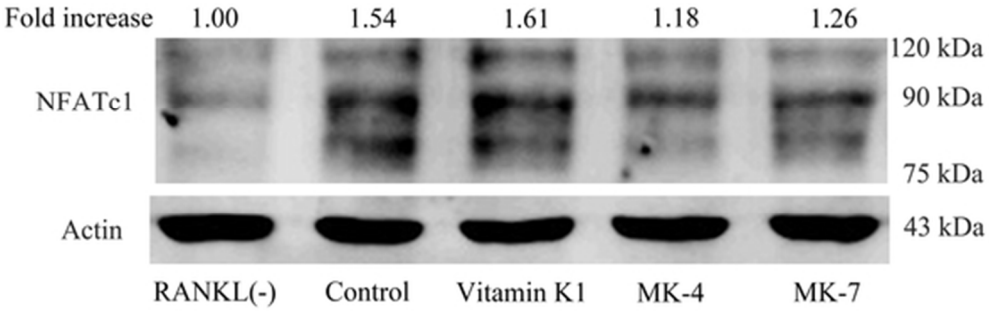

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BMMs were pretreated with or without vitamin K species (20 μM) and further stimulated with RANKL (50 ng/ml) for 3 days. Actin was used as the internal control.
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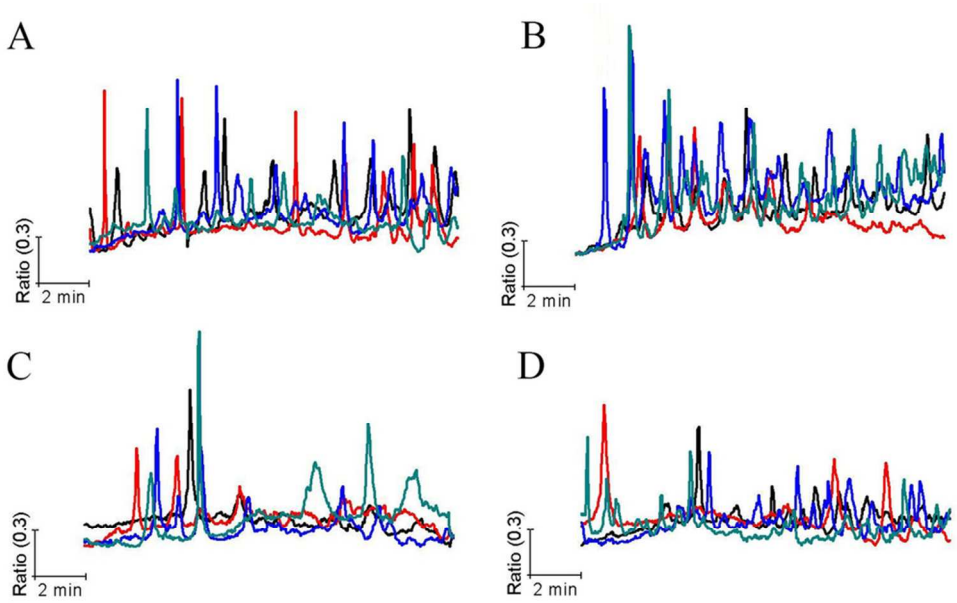

Fig. 5 Effect of vitamin K species on RANKL-induced [Ca2+]i oscillation.
BMMs were incubated with or without vitamin K (20 μM) in the presence of RANKL (50 ng/ml) for 24 h. (A) Control; (B) Treated with vitamin K1; (C) Treated with MK-4; (D) Treated with MK-7.
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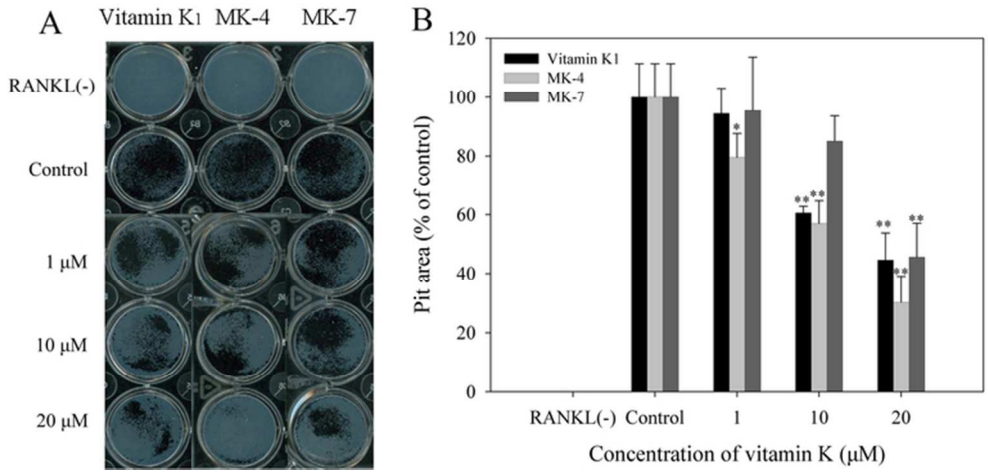

Fig. 6 Vitamin K species inhibit the bone resorption activity of osteoclasts.
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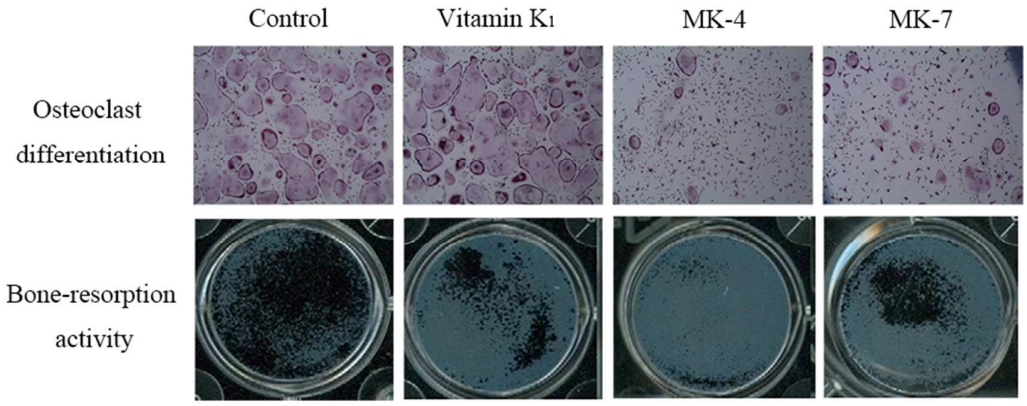

Vitamin K_1, MK-4 and MK-7 have anti-osteoporotic properties, while their regulation manners on osteoclastogenesis are somewhat different.