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A comparatively study of menaquinone-7 isolated from *Cheonggukjang* with vitamin K_1 and menaquinone-4 on osteoblastic cells differentiation and mineralization

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ABSTRACT

The effect of menaquinone-7 isolated from *cheonggukjang* was comparatively investigated with vitamin K_1 and menaquinone-4 on cell differentiation and mineralization of the osteoblastic cell line MC3T3-E1. Results indicated that all vitamin K species significantly increased MC3T3-E1 cell proliferation, cellular alkaline phosphatase activity, osteocalcin synthesis, and calcium deposition in a dose-dependent manner. Menaquinone-4 and menaquinone-7 had more potent effects on calcium deposition than vitamin K_1, and their effects were only partly reduced by warfarin (γ-carboxylation inhibitor) treatment, while warfarin abolished the induction activity of vitamin K_1 on calcification. This suggests that vitamin K_1 and K_2 (menaquinone-4 & menaquinone-7) may have different mechanisms in stimulating osteoblast mineralization. In addition, the mRNA expression ratio of osteoprotegerin and the receptor activator of nuclear factor-kB ligand was also dramatically increased by treatment with vitamin K_1 (62%), menaquinone-4 (247%), and menaquinone-7 (329%), suggesting that vitamin K may suppress the formation of osteoclast by up-regulating the ratio of osteoprotegerin/receptor activator of nuclear factor-kB ligand in osteoblasts. These results provide compelling evidence that vitamin K_1, menaquinone-4, and menaquinone-7 all can promote bone health, which might be associated with elevations in the osteoprotegerin/receptor activator of nuclear factor-kB ligand ratio.

1. Introduction

Bone balance required a dynamic balance between bone resorption and formation. Osteoporosis is defined as bone resorption exceeds bone formation, resulting in low bone mineral density and an increased risk of fractures (Li et al., 2012; Maeda et al., 2001). Our previous research demonstrated that vitamins K_1 and K_2 have a direct inhibitory effect on osteoclastic bone resorption (Wu et al., 2015), and the administration of *cheonggukjang* containing high level of vitamin K_2 is helpful for osteoporosis prevention in ovariectomized rats (Wu et al., 2014).

Natural vitamin K has two different types: vitamin K_1 (phylloquinone) and K_2 (menaquinone). Vitamin K_1 is mainly existed in green vegetables, while vitamin K_2 distributes in fermented food, especially in fermented soybean (Wu and Ahn, 2011). According to the length of isoprene units at the 3-position of naphthoquinone, menaquinone (vitamin K_2) can be divided into several species. Menaquinone-4 (MK-4) and menaquinone-7 (MK-7) with four and seven isoprene units respectively have been commonly used as nutritional supplement (Yamaguchi and Weitzmann, 2011).

Although vitamin K has been reported to have bone-protective activity, the molecular mechanism is poorly defined (Atkins et al., 2009; Wu et al., 2014). Vitamin K plays a principal role in the conversion of glutamic acid residues into γ-carboxyglutamic acid (Gla) in Gla-containing proteins (Lee et al., 2007). The ratio between under-carboxylated osteocalcin (ucOC) and γ-carboxylated osteocalcin (cOC) is associated with bone mineral density and hip fracture risk, while vitamin K treatment can effectively increase cOC levels and inhibit bone loss (Tsukamoto, 2004; Tsukamoto et al., 2000). In contrast, a recent

Abbreviations: ALP, alkaline phosphatase; α-MEM, α-minimum essential medium; cOC, γ-carboxylated osteocalcin; ESI, electrospray ionization; FBS, fetal bovine serum; Gla, γ-carboxyglutamic acid; MK-4, menaquinone-4; MK-7, menaquinone-7; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor-kB ligand; ucOC, under-carboxylated osteocalcin

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clinical study of healthy postmenopausal women indicated that vitamin K treatment significantly reduces ucOC ratio, but does not alter bone turnover, density, or geometry (Binkley et al., 2009). These results are contradictory, and a direct cause-effect relationship between osteocalcin γ-carboxylation and the effects of vitamin K species on bone metabolism remain unclear (Yamaguchi and Weitzmann, 2011). It has been suggested that vitamin K_1 and MK-4 stimulate the proliferation and function of osteoblastic cells (Akedo et al., 1992; Atkins et al., 2009; Zhang et al., 2016), while MK-7 has been reported to regulate osteoblast-specific gene expression and alkaline phosphatase (ALP) activity in MC3T3-E1 cells (Katsuyama et al., 2005, 2007; Yamaguchi and Weitzmann, 2011). However, the effects of vitamin K_1, MK-4, and MK-7 on osteoblastic MC3T3-E1 cell differentiation and mineralization have not been comparatively studied.

The purpose of this study was to compare and investigate the abilities of three vitamin K species (vitamin K_1, MK-4 and MK-7) to stimulate osteoblast cell proliferation, differentiation and mineralization, and to determine their relationships with γ-carboxylation.

2. Materials and methods

2.1. Chemicals and reagents

Menaquinone-7 was purified (97% purity) in our laboratory from *cheonggukjang*, a Korean traditional fermented food which was manufactured according to a previous reported method (Wu and Ahn, 2011). Briefly, *cheonggukjang* was mixed with 1.2-fold of 2-propanol and 2.4-fold of *n*-hexane, and subject to vigorous shaking for 1 min, after which the mixture was allowed to stand for 1 h. After 1 h, the hexane layer was withdrawn and concentrated under reduced pressure. The oil residue was 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-method solvent system (100:0, 75:25, 50:50, 25:75, 0:100; each 200 mL, v/v) to yield purified MK-7. The isolated and purified MK-7 was verified by Waters Xevo TQD UPLC-MS/MS and the stock was frozen at −70 °C for further study. Vitamin K_1 (phylloquinone) and menaquinone-4 (MK-4) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Warfarin was obtained from Cayman Chemical (Ann Arbor, MI, USA). Penicillin/streptomycin and α-minimum essential medium (α-MEM) were obtained from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from HyClone (South Logan, Utah, USA). All of the other chemicals used in this study were of analytical grade or complied with the standards required for cell culture experiments.

2.2. UPLC-MS/MS analysis

The purified sample was injected to a quadrupole UPLC-MS/MS system (Waters, Milford, MA, USA) with a Waters Xevo™ TQD mass spectrometer coupled with an API interface, and furnished with an electrospray ionization (ESI) source operating in positive mode. Separations were performed on Waters Acquity UPLC BEH amide column (2.1 mm × 100 mm, 1.7 μm, Waters, Milford, MA, USA). The column temperature was maintained at 25 °C, and the full loop injection volume of the autosampler was 10 μL. The mobile phase A and B consisted of 0.1% formic acid in distilled water and 0.1% formic acid in methanol: acetonitrile (40:60, v/v), respectively. MK-7 was eluted according to a linear gradient mode (B 100% from 0 to 0.5 min, B from 100 to 50% from 0.5 to 4 min, B 50% from 4 min to 4.5 min, B from 50 to 100% from 4.5 to 6 min, B 100% from 6 to 10 min) at a flow rate of 0.5 mL/min. Compound UV spectra was acquired at the wavelength of 248 nm. The quadrupole mass spectrometer was set to scan the *m/z* range of 100–1000. The desolvation temperature was set at 350 °C and the source temperature at 150 °C. The capillary voltage was set at 2 kV. Nitrogen was used as desolvation gas and cone gas with flow rates of 500 L/h and 30 L/h, respectively.

2.3. Cell culture

The pre-osteoblastic cell line MC3T3-E1 subclone 4 (CRL-2593) cells derived from neonatal mouse calvariae were purchased from the American Type Culture Collection (Manassas, VA, USA) as an *in vitro* model for osteoblast metabolism (Katsuyama et al., 2007), and cultured following the instructions previously described (Yamaguchi and Weitzmann, 2011). Briefly, cells were cultured in α-MEM containing 10% FBS and antibiotics (100 U/mL penicillin G, 100 μg/mL streptomycin) at 37 °C and 5% CO_2 in a humidified incubator. Cells were subcultured every 2–3 days with 0.25% trypsin containing EDTA (HyClone).

2.4. Proliferation assay

MC3T3-E1 cells were seeded in 96-well plates at a density of 8×10^3 cells/well and cultured overnight. Then, vitamin K or vehicle was applied to treat the cells. After 3 days of culturing, a colorimetric MTT [3-(4,5-dimethylthiazol-2-yl)-2,5diphenyl tetrazolium bromide] assay was performed to evaluate the proliferation of MC3T3-E1 cells (Li et al., 2012).

2.5. Osteoblast differentiation and ALP activity assay

MC3T3-E1 cells were seeded and cultured for 24 h in α-MEM (2 mL/well) containing 10% FBS in a 24-well plate at a density of 5×10^4 cells/well. When the cells reached confluence, the medium was aspirated and changed with mineralization medium [α-MEM containing 10% FBS, 10 mM β-glycerophosphate and 50 μg/mL L-ascorbic acid] as previously reported (Maeda et al., 2001). Vitamin K species or vehicle was added at doses of 0.1, 1, or 10 μM in the presence or absence of warfarin, and the medium was refreshed every 3 days. At differentiation days 3, 7, and 14, the cells were rinsed with PBS and sonicated for 1 min in 0.1 M Tris buffer, pH 7.2, containing 0.1% Triton X-100. The ALP activity of osteoblastic cells was determined with a LabAssay ALP kit (Wako Chemical, Osaka, Japan) according to the manufacturer's directions.

2.6. Detection of osteocalcin (OC)

The ucOC and cOC levels of the MC3T3-E1 cells were measured as previously described (Li et al., 2012), with minor modification. A 0.7 mL of the MC3T3-E1 cell suspension at a density of 2×10^4 cells/well was plated into each well of a 48-well plate. The mineralization medium containing different doses of vitamin K species (0.1, 1, or 10 μM) was changed once every 3 days. After 12 days of incubation, the supernatant from the culture was collected, and the contents of ucOC and cOC were measured using MK129 mouse Glu-osteocalcin and Gla-osteocalcin High Sensitive EIA kits (TaKaRa, Tokyo, Japan). ELISA assays for ucOC and cOC detection were carried out according to the manufacturer's instructions and the absorbance was measured on a microplate reader (BMG Labtech, Offenburg, Germany) at a wavelength of 450 nm.

2.7. Measurement of calcium levels

Osteoblastic cells were plated in a 48-well plate at a density of 1.2×10^4 cells/well in 0.8 mL of mineralization medium containing different doses of vitamin K species (0.1, 1, or 10 μM) in the presence or absence of warfarin. After 18 days of culturing, the calcium levels in the cell monolayers were measured using the cresolphthalein method as described previously (Findlay et al., 2004), with minor modification. The cultures were first washed with Ca^{2+}-free PBS 3 times, and then the calcium was extracted using 0.6 m HCl and sonicated for 10 min. After centrifugation at 10,000 × *g* for 10 min, the calcium levels were measured by colorimetric assay using a Calcium Detection Kit (Abcam,

Cambridge, UK), according to the manufacturer's instructions. The absorbance at 575 nm was measured using a POLARstar optima microplate reader (BMG Labtech, Offenburg, Germany).

2.8. Real-time PCR quantification of gene expression

MC3T3-E1 cells (4×10^5 cells/well) were treated with vitamin K species (10 μM) for 24 h in a 6-well plate, and the total RNA was isolated with RiboEx reagent (GeneAll Biotechnology, Seoul, Korea) according to the manufacturer's instructions. The isolated RNA was submitted to Biospec-nano spectrophotometer (A116453, Shimadzu, Kyoto, Japan) to evaluate its quality and quantity. First-stranded cDNA was synthesized from 1.5 μg of total RNA with the first-strand cDNA synthesis kit (TaKaRa, Tokyo, Japan). Thereafter, the cDNA was amplified using the following primer sets: OPG, 5′-CAA TGG CTG GCT TGG TTT CAT AG-3′ (forward) and 5′-CTG AAC CAG ACA TGA CAG CTG GA-3′ (reverse); RANKL, 5′-CAT GTG CCA CTG AGA ACC TTG AA-3' (forward) and 5′-CAG GTC CCA GCG CAA TGT AAC-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) (Zhang et al., 2010). Quantitative real-time PCR was performed using the SYBR Green qPCR kit (Enzynomics, Daejeon, Korea) and the Rotor Gene (model RG-6000; Corbett Research, Sydney, Australia). The relative expression levels of OPG and RANKL were normalized to GAPDH and compared to the control group.

2.9. Statistical analysis

Data analyses were performed with SAS System Edition for Windows version 9.1 (SAS Institute Inc., Cary, NC, USA), and significant differences between groups were analyzed by Duncan's multiple comparisons ($p < 0.05$).

3. Results

3.1. Verification of biosynthesized MK-7

The biosynthesized chemical was purified by silica open column and verified by UPLC-MS/MS. Results indicated that the retention time of the authentic standard of menaquinone-7 was 0.53 min in UPLC-MS/MS (Fig. 1), and the biosynthesized chemical also showed the same retention time. Furthermore, the same quasi-molecular ion peaks were obtained at *m/z* 650 $[M+H]^+$. As a result, the biosynthesized chemical isolated from *cheonggukjang* was assigned as menaquinone-7 (MK-7).

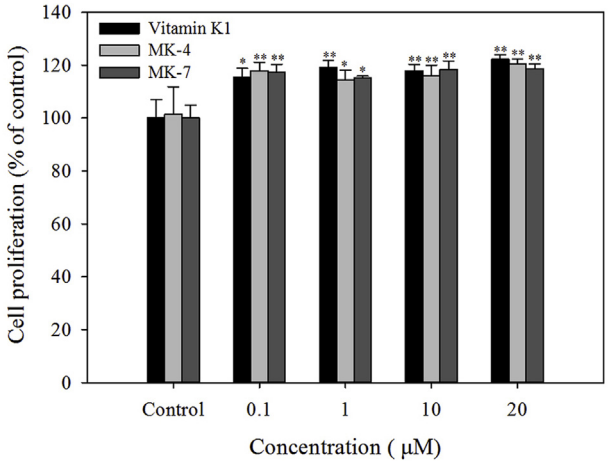

Fig. 2. Effect of vitamin K species on the proliferation of MC3T3-E1 cells. The cells were treated with vitamin K1, MK-4, and MK-7 at different concentration (0.1 μM, 1.0 μM, 10 μM, and 20 μM, respectively) for 3 days, and cell proliferation was measured by MTT assay. Data were shown as mean ± SD of three independent experiments. Significant difference *p < 0.05, **p < 0.01 vs Control (treated with same amount of vehicle).

3.2. Effect of vitamin K on cell proliferation

To investigate the effects of vitamin K_1, MK-4, and MK-7 on the proliferation of MC3T3-E1 cells, different concentrations (0.1, 1, 10, and 20 μM) of vitamin K species were added to the culture media for 3 d. The proliferation results of MC3T3-E1 cells determined by MTT assay, are as shown in Fig. 2. The average absorbance values are directly related to the viable cell numbers, which were expressed as a percentage of the control. Vitamin K_1, MK-4, and MK-7 treatments all

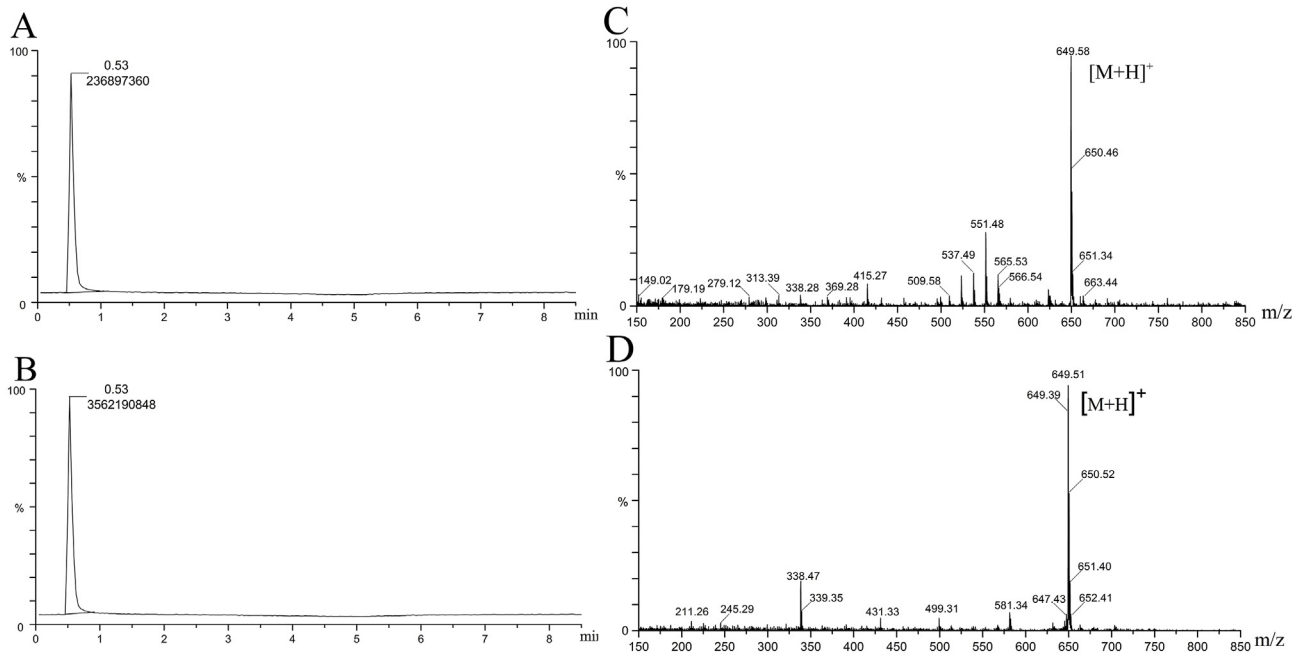

Fig. 1. Total ion chromatogram of authentic standard of MK-7 and biosynthesized MK-7 by B. *subtilis* BY07 and their fragmentation patterns by UPLC-MS/MS. (A), standard of MK-7; (B), biosynthesized MK-7; (C), mass spectra of MK-7; (D), mass spectra of biosynthesized MK-7.

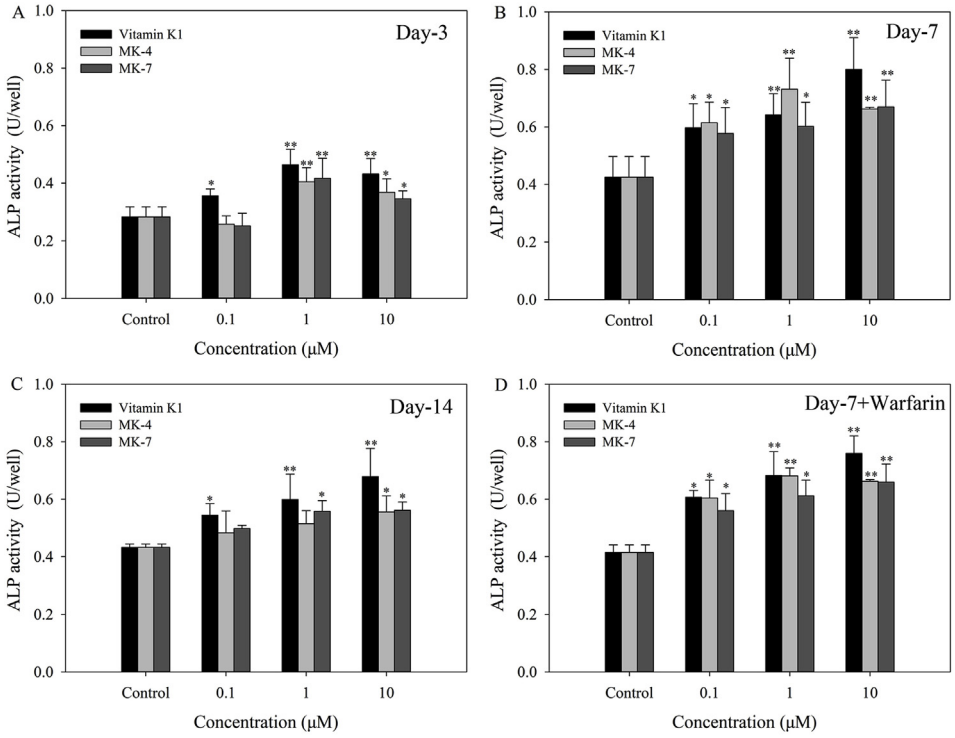

Fig. 3. Effect of vitamin K species on alkaline phosphatase (ALP) activity of MC3T3-E1 cell culture. The cells were treated with vitamin K1, MK-4, and MK-7 at different concentration (0.1 μM, 1.0 μM, and 10 μM respectively) in mineralization medium for 3 (A), 7 (B), and 14 (C) days. Vitamin K and 20 mM warfarin were simultaneously added to the mineralization medium culture for 7 days (D). Data were shown as mean ± SD of three independent experiments. Significant difference $*p < 0.05$, $**p < 0.01$ vs Control (treated with same amount of vehicle).

significantly increased the proliferation of these cells by up to 120% of the basal value of the vehicle control. Moreover, even high doses of vitamin K species (20 μM) did not exert any cytotoxic effect on MC3T3-E1 cell growth.

3.3. Effect of vitamin K on ALP activity

To evaluate the effects of vitamin K species on the differentiation of MC3T3-E1 cells, the ALP activities during day 3–14 were determined by an ALP assay kit, and the results were shown in Fig. 3ABC. The ALP activity increased during the initial stage of differentiation (days 3–7), and then plateaued during the second week. Both vitamin K_1 and vitamin K_2 (MK-4 & MK-7) significantly promoted ALP activity during the differentiation course from day 3 to day 14 (Fig. 3ABC). In particular, the vitamin K_1 group exhibited a much higher stimulatory effect than the vitamin K_2 groups (MK-4 and MK-7) on ALP expression, with vitamin K_1, MK-4, and MK-7 increasing the ALP activity by 90%, 56%, and 58%, respectively, compared with the control on day 7 (Fig. 3B).

It has been reported that the γ-carboxylation effect of vitamin K could be inhibited by warfarin (Atkins et al., 2009). Thus, to investigate the involvement of the γ-carboxylation effect of vitamin K_1, MK-4, and MK-7, osteoblastic cells were mineralized in the presence or absence of warfarin at a concentration of 20 μM. However, the results indicated that the addition of warfarin did not suppress the stimulatory effects of all vitamin K species on ALP activity (Fig. 3D), implying that the mechanisms of vitamin K-induced ALP expression is γ-carboxylation-independent.

3.4. Effect of vitamin K on OC expression

The extent of osteocalcin accumulation in the culture medium was determined after the cells were treated with 10 μM of vitamin K species for 12 days in the presence or absence of warfarin (20 μM). As shown in Table 1, without warfarin, the ucOC content was significantly reduced after the addition of MK-7, while the cOC content was remarkably increased in culture medium with the addition of vitamin K_1, MK-4 and MK-7. Warfarin treatment significantly reduced the levels of cOC within all groups. Additionally, no significant difference in ucOC content was determined between the vitamin K groups and the control groups in the presence of warfarin. The γ-carboxylation function of vitamin K_1, MK-4, and MK-7 were all dramatically inhibited by warfarin treatment. In particular, the γ-carboxylation-stimulating effects of vitamin K_1 were almost abolished. Vitamin K_2 (MK-4 and MK-7) was less sensitive to warfarin, which still stimulated OC γ-carboxylation to a greater extent than the control, although this effect was remarkably reduced by warfarin (Table 1).

3.5. Calcium deposition assessment

Cell matrix-associated calcium was significantly increased by the vitamin K analogs treatment in a dose-dependent manner (Fig. 4). At the same dose of treatment (10 μM), the vitamin K_2 groups (MK-4 & MK-7) exhibited a remarkably higher stimulatory effect on calcium deposition in MC3T3-E1 cells. The calcium levels of vitamin K_1 (Fig. 4A), MK-4 (Fig. 4B), and MK-7 (Fig. 4C) were increased up to 144%, 166%, and 167%, respectively. The vitamin K antagonist warfarin (5 and 20 μM) was added to evaluate the γ-carboxylation function of vitamin K species. Fig. 4A showed that in MC3T3-E1 cells, vitamin K_1–induced calcium deposition was inhibited by warfarin treatment, indicating that the efficiency of vitamin K_1 to stimulate calcium deposition is associated with its γ-carboxylation activity. However, both MK-4 (Fig. 4B) and MK-7 (Fig. 4C) were partially sensitive to warfarin, suggesting that vitamin K_2-induced γ-carboxylation is only partially suppressed by warfarin or is γ-carboxylation-independent. Moreover, it was interesting to observe that high doses of warfarin may accelerate calcification (Fig. 4ABC), a phenomenon that was verified by treatment with warfarin alone at different concentrations without vitamin K treatment (Fig. 4D).

3.6. Effect of vitamin K on OPG and RANKL gene expression

To determine whether vitamin K species could influence osteoclast differentiation by modulating MC3T3-E1 cytokine expression, the expression of OPG and RANKL mRNA in MC3T3-E1 osteoblasts was

Table 1
Effect of vitamin K species and warfarin on accumulation of osteocalcin in extracellular medium.

Treatments	Under-carboxylated osteocalcin (ng/mL)		γ-Carboxylated osteocalcin (ng/mL)	
	Warfarin (−)	Warfarin (+)	Warfarin (−)	Warfarin (+)
Control	0.230 ± 0.052	0.202 ± 0.004	0.434 ± 0.008	0.395 ± 0.006$^{\#\#}$
Vitamin K_1	0.213 ± 0.008	0.195 ± 0.030	0.477 ± 0.010**	0.412 ± 0.005$^{\#\#}$
MK-4	0.200 ± 0.022	0.187 ± 0.012	0.501 ± 0.021**	0.451 ± 0.024**$^{\#\#}$
MK-7	0.186 ± 0.012*	0.182 ± 0.001*	0.530 ± 0.005**	0.484 ± 0.001**$^{\#\#}$

The cells were treated with vitamin K_1, MK-4, and MK-7 at a concentration of 10 μM in mineralization medium for 12 days, with or without warfarin (20 μM) treatment. Data were shown as means ± SD of three independent experiments. Significant difference *$p < 0.05$, **$p < 0.01$ vs Control in the same column, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with warfarin (−) in the same row.

analyzed by Real-Time PCR. The results in Fig. 5 indicated that vitamin K_2 (MK-4 & MK-7) significantly increased OPG mRNA expression after 24 h of treatment at a concentration of 10 μM (Fig. 5A). Meanwhile, the expression of RANKL mRNA was slightly reduced by treatment with vitamin K species, although no statistically significant differences were observed (Fig. 5B). Overall, the ratio of OPG/RANKL mRNA expression in the vitamin K_1, MK-4, and MK-7 treatment groups were increased by 62%, 247%, and 329%, respectively, as compared with the vehicle control (Fig. 5C).

4. Discussion

Vitamin K species play an important role in bone metabolism, and oral supplementation of vitamin K has been considered to be a therapeutic tool for osteoporosis (Kameda et al., 1996; Villa et al., 2017; Wu et al., 2014; Yamaguchi et al., 2003). However, the specific mechanisms of vitamin K species in promoting the bone formation are still not well-defined. Therefore, a comparative study on the direct effects of vitamin K_1, MK-4, and MK-7 on osteoblastic MC3T3-E1 cell differentiation and mineralization was performed in the present study. The phenotype of osteoblast is specifically divided into two stages: during the first stage, the matrix matures and specific proteins such as ALP are synthesized; while during the second stage, the matrix becomes mineralized and late-stage bone markers such as cOC are produced (Cho et al., 2010).

Results of the present study indicated that vitamin K_1, MK-4, and MK-7 all demonstrated an enhanced osteogenic effect in osteoblastic MC3T3-E1 cells by significantly increasing osteoblastic cell proliferation and the activity of the bone formation marker ALP during the first stage of the osteoblast phenotype. During the initial proliferation period (3 days), vitamin K_1, MK-4, and MK-7 increased cell proliferation to up to 120% of the basal value of the vehicle control (Fig. 2), indicating that vitamin K species had an anabolic effect on bone matrix formation by stimulating the growth rate of the osteoblastic cells. These results are different from those of a previous study, which reported that MK-4 suppressed MC3T3-E1 cell proliferation (Akedo et al., 1992). The difference may be due to different culture medium utilized: α-MEM containing 0.1% fetal bovine calf serum (FCS) was used in that previous study, while 10% FBS was used in the present study. ALP is associated with bone metabolism and osteoblasts differentiation, its activity is considered to be one of the most common biochemical markers of osteoblast differentiation and osteogenic properties (Li et al., 2012). Within the span of osteoblast growth and differentiation, ALP is expressed immediately following the cell proliferation period and then during osteoblast differentiation (Cho et al., 2010). In this study, ALP activity on days 3, 7, and 14 were assessed. During the entirety of the culture course, treatment with the vitamin K species all resulted in dramatically increased ALP activity in comparison to the control groups (Fig. 3ABC). These findings are consist with previous results that vitamin K increased ALP activity in MC3T3-E1 cells (Akedo et al., 1992; Yamaguchi et al., 2001) as well as in rats (Sogabe et al., 2007).

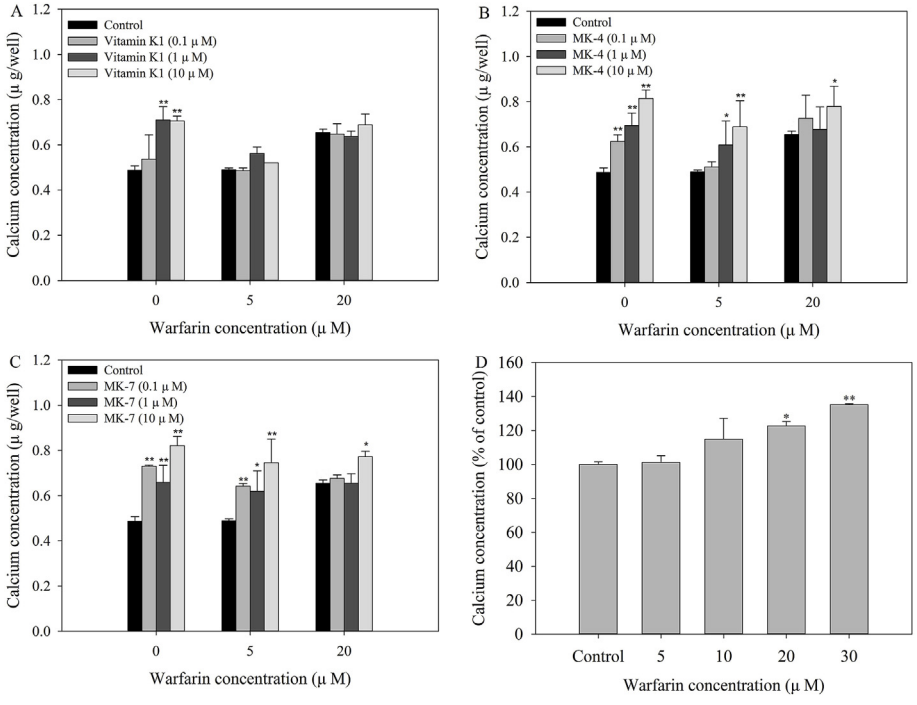

Fig. 4. Effect of vitamin K species and warfarin on calcium deposition in MC3T3-E1 cells. Cells were cultured under mineralizing conditions for up to 18 days either untreated or in the presence of vitamin K_1 (A), MK-4 (B), or MK-7 (C) at 0.1, 1, 10 μM and in the presence or absence of warfarin at concentration of 5 or 20 μM. D: cells were cultured with different dose of warfarin (5, 10, 20, 30 μM) for 18 days. Cell monolayers were assayed for calcium deposition. Values are means ± SD of three independent experiments. Significant difference *$p < 0.05$, **$p < 0.01$ vs Control.

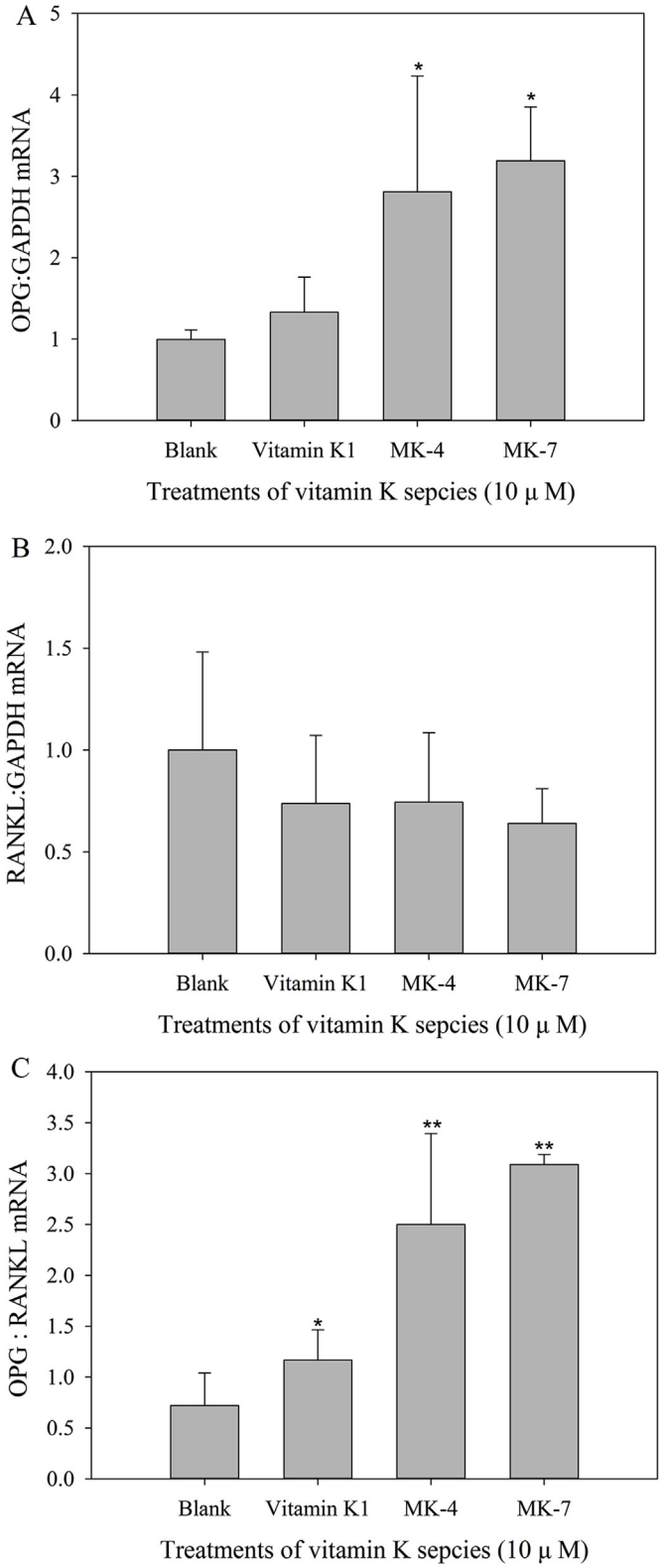

Fig. 5. Effect of vitamin K species on OPG and RANKL mRNA expression in MC3T3-E1 cells over 24 h. Expression of individual genes was normalized to GAPDH mRNA levels. Values are mean ± SD of triplicate reactions. Significant difference $*p < 0.05$, $**p < 0.01$ compared with vehicle control.

Compared with the vitamin K_2 groups (MK-4 & MK-7), vitamin K_1 exhibited a much greater stimulatory effect on ALP activity, with a two-fold increase over the basal control value on day 7 (Fig. 3B). Treatment with warfarin (20 μM), a vitamin K antagonist and γ-carboxylase inhibitor, did not inhibit the ALP-promoting effects of vitamin K_1, MK-4, and MK-7 (Fig. 3D), indicating that the mechanisms of vitamin K-induced ALP expression are γ-carboxylation-independent.

The expression of OC is a late-stage osteoblast differentiation marker (Li et al., 2012). In this study, the levels of ucOC in the culture medium were significantly reduced by MK-7 treatment, while the levels of cOC were all dramatically increased after treatment with vitamin K_1, MK-4, and MK-7 (Table 1). Recent study also indicated that MK-7 could reduce ucOC content in postmenopausal women, and prevent age-related deterioration of trabecular bone microarchitecture at the tibia (Rønn et al., 2016). These results offer further support for the important role of vitamin K in the γ-carboxylation of OC. When treated with warfarin, the vitamin K-dependent cOC levels were remarkably reduced, especially for vitamin K_1. No statistical difference was observed compared to control, implying that vitamin K_1-dependent γ-carboxylation was abolished by warfarin. These results are consist with that of a previous report which examined the regulatory effect of warfarin on vitamin K_1-dependent γ-carboxylation (Wallin and Hutson, 2004). However, vitamin K_2 (MK-4 and MK-7) was less sensitive to warfarin. Although γ-carboxylation was significantly suppressed by warfarin treatment, vitamin K_2 still increased the cOC levels compared to the control (Table 1), suggesting that vitamin K_2-dependent γ-carboxylation is not completely inhibited by warfarin.

Enhancing the mineralization of osteoblasts is the ultimate aim of any therapy targeting bone regeneration (Li et al., 2012). Because osteoblastic mineralization occurs through the deposition of calcium in the extracellular matrix (Cho et al., 2010), the calcium deposition levels were examined for further investigation. The results showed that vitamin K species all significantly increased the calcium levels in MC3T3-E1 cell layers in a dose-dependent manner (Fig. 4ABC). Vitamin K_1 (Fig. 4A) was the least potent inducer of calcification, and its activity was abolished by the γ-carboxylation inhibitor warfarin. This result is in accordance with that of a previous study, which also reported that vitamin K_1-induced mineralization was dependent on the γ-carboxylation pathway and was abolished by warfarin (Atkins et al., 2009). MK-4 (Fig. 4B) and MK-7 (Fig. 4C) had more potent effects on calcium deposition, which was only partly reduced by warfarin treatment and was consistent with the cOC results described above. These findings suggested that vitamin K_1, MK-4, and MK-7 differ in their mode or extent of action on stimulating osteoblastic bone formation. Additionally, our previous studies reported that vitamin K_2 inhibited the RANKL-induced osteoclast-like cell formation, while same dose of vitamin K_1 had no effects (Wu et al., 2015). However, the structural differences among vitamin K_1, MK-4, and MK-7 only exist in their side chain, which indicates that the side chains of vitamin K may play an important role in bone metabolism regulation. Interestingly, warfarin at high dose (20–30 μM) also caused an acceleration of calcification in MC3T3-E1 cells (Fig. 4). However, this effect was not observed in a similar study (Atkins et al., 2009), which was carried out using normal human bone-derived cells (NHBC). In some other studies, high doses of warfarin have been reported to cause the excessive matrix mineralization of hypertrophic chondrocytes (Yagami et al., 1999), as well as the rapid calcification of the elastic lamellae in rat arteries and heart valves (Price et al., 1998).

Bone is constantly resorbed by osteoclasts and replaced by osteoblasts in a dynamic balance called bone remodeling. During this remodeling process, OPG and RANKL from osteoblasts have been reported to be key cytokines in regulating osteoclast differentiation (Lamghari et al., 2006; Li et al., 2012). RANKL plays a crucial role in the activation of osteoclasts by binding to its receptor RANK and then stimulating the functional signal transduction of osteoclasts (Li et al., 2012). However, this stimulatory effect of RANKL could be neutralized by OPG, which competes against RANK and inhibits the interaction between RANKL and its receptor RANK, thus inhibiting the differentiation of osteoclasts as well as the activation of mature osteoclasts

(Kostenuik, 2005; Li et al., 2012). Generally speaking, the balance between OPG and RANKL is critical to the regulation of bone remodeling, and the relative expression ratio of OPG to RANKL can reflect the etiology of bone disease or the compensatory responses by the human body (Kostenuik, 2005). In the present study, the expression of OPG mRNA was remarkably increased after treatment with MK-4 or MK-7 for 24 h, as compared to the control and vitamin K_1 (Fig. 5A). However, the expression of RANKL mRNA in all vitamin K species-treated groups was slightly decreased, but no statistical difference was observed when compared with control (Fig. 5B). Overall, the mRNA expression ratio of OPG/RANKL was statistically increased by treatment of vitamin K_1, MK-4, and MK-7, with the effect of MK-7 (329%) > MK-4 (247%) > vitamin K_1 (62%) (Fig. 5C). Additionally, our previous study proved that vitamin K_1, MK-4, and MK-7 all effectively inhibited the RANKL-induced osteoclastic bone resorption in a dose dependent manner (Wu et al., 2015). The results from this study are consistent with previous studies which observed that vitamins K_1 and MK-4 inhibited osteoclast formation in human bone marrow culture systems, accompanied with the decreased expression of RANKL mRNA and the enhanced expression of OPG mRNA (Koshihara et al., 2003). Moreover, Atkins et al. (2009) reported that vitamin K species increased the mRNA ratio of OPG/RANKL in MLO-Y4 cells and in human cultures. Briefly, these observations indicated that vitamin K species positively modulate bone metabolism, possibly by up-regulating the ratio of OPG/RANKL, which mediates suppressive effect on osteoclastogenesis and leads to bone regeneration (Li et al., 2012).

Briefly, vitamin K_1, MK-4, and MK-7 may all capable of stimulating osteoblastic bone regeneration by increasing cell proliferation, cellular ALP activity, cOC synthesis, and calcium deposition, as well as by up-regulating the ratio of OPG/RANKL expression. However, vitamin K_2 had a greater effect on stimulating calcium deposition, was less sensitive to warfarin, and was more potent in positively regulating the OPG/RANKL expression ratio. It is possible that vitamin K_2 (especially MK-7 from *cheonggukjang*) could be a promising supplement for the prevention and treatment of osteoporosis.

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Appendix A. Supplementary data

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Transparency document

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