Effect of Vitamin K2 as A Stimulants of Bone Regeneration in Osteoporosis Therapy

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Introduction: Osteoporosis stems from misbalance between bone forming and bone resorption, which lead to increased risks of bone fractures. In recent years, stem cell therapy introduced as a promising strategy for bone regeneration in osteoporosis due to their bone regeneration potential. However, stem cells require different stimulator to accelerate bone regeneration and repair processes. Previous Studies showed that Vitamin K2, as an osteoprotective factor, promote and inhibit proliferation and activity osteoblast and osteoclasts cell line, respectively. We aimed to elucidate effect vitamin K2 on dental pulp stem cells (DPSCs) proliferation and their differentiation into osteoblast, and evaluation effect of this vitamin on the process of differentiating peripheral blood mononuclear cells (PBMNCs) into osteoclast and the activity of these cells. **Material and Methods:** DPSCs and PBMNCs were used for induction towards the osteoblast and osteoclasts, respectively in the presence of various concentrations of vitamin K2. Cell viability was assessed by MTT assay. Osteogenesis assayed by alizarin red S staining and osteogenic gene expression as well as osteoclastogenesis by tartrate-resistant acid phosphatase (TRAP) staining, Annexin V/PI assay, pit formation and NF-κB gene expression. **Results:** Our data showed that vitamin K2 at a concentration of 10μM increased both proliferation and osteogenesis activities of DPSCs and also increased the incidence of apoptosis in TRAP-positive cells as well as decrees in an expression of NF-κB and pit formation. **Conclusion:** These results suggest that simultaneous use of vitamin K2 and DPSCs can be a purpose of stem cell therapy in osteoporosis and conducting further pre-clinical studies.

Keyword: Dental-pulp derived stem cells; Vitamin K2; Osteoporosis therapy ; Bone regeneration

Introduction

Aging of the population worldwide is associated with a remarkable increase in the incidence of bone diseases, including osteoporosis, osteoarthritis. The international osteoporosis foundation reported that osteoporosis diagnosed in over 200 million people worldwide, and also most of this population is postmenopausal women (1). Therefore, osteoporosis, as a public health concern, is represented by an increase in bone fracture and limitation in performing daily activities (2, 3).

Pathophysiological studies defined that this disease stems from a misbalance between bone forming capacity, mediated by declines in the number and function of osteoblasts, and bone resorption carried out by the osteoclast(1, 2, 4). Other studies about osteoporosis development demonstrated that mesenchymal stem cells (MSCs) differentiate into adipocyte rather than osteoblast (5-7). For treatment and prevention of osteoporosis, the variety of medication is prescribed that improve bone anabolism and or inhibit bone resorption, such as bisphosphonate, calcitonin and parathyroid hormone (1, 5, 8). One of the main drawbacks of these drugs is the inability to repair and regeneration of bone tissue (1, 2).

In the recent decade, stem cell therapy introduces a promising strategy to bone regeneration in osteoporosis (1, 2, 9). Various sources of stem cells have been evaluated to achieve this aim, such as embryonic stem cells, induced pluripotent stem cells and mesenchymal stem cells (MSC) (1, 10, 11). Among these cells, MSCs characterized by the several advantages, such as self-renewal potential, tri-lineage differentiation, immunologically privileged property, migration to site of injury and easily isolate in large quantities from the patient themselves, also the therapeutic delivery of MSCs to tissue regeneration has

been approved by U.S. Food and drug administration (12-15).

However, the clinical use of MSCs in osteoporosis treatment is associated with concerns, including efficiency homing of MSCs to bone tissue, the long-term survival and differentiation capacity into osteoblast (7). Although, the several studies have been performed to improve this characterization of MSCs through within-cell (genetic modification) or outside the cell (adjusting external factors), but clinical application of these modifications requires more studies in terms of preclinical and clinical level (5, 16-18).

Vitamin K, as a group of fat-soluble vitamins, is an essential factor in blood coagulation and bone formation mammals (19-23). Vitamin K naturally occurs in two forms: phylloquinone (K1) and menaquinones (K2) (24-26). The most common form of K2, menaquinone 4 (MK4), has a greater osteoprotective effect (25).

The previous studies showed that vitamin K promotes osteoblast cell lines (such as MG63, MC3T3-E1, COS-7, etc) for proliferation and differentiation into osteoblast through the expression of osteoblastic markers including alkaline phosphatase (ALP), insulin-like growth factor-1, growth differentiation factor-15, stanniocalcin 2, Osterix, and RUNX2 (25-28). Moreover, some studies indicated that vitamin K inhibits osteoclast cell lines (such as RAW264.7) activity by reducing the receptor activator of nuclear factor kappa-B ligand (NF-κB), a transmembrane protein that has proved to be an essential molecule for osteoclastogenesis (16-18).

Despite previous in vitro and pre-clinical studies, Yonf Fong and colleagues performed a meta-analysis study to the evaluation of effects of vitamin K on bone mineral density (BMD, as bone formation marker) in the randomized controlled clinical trial. This study showed that treatment effects of vitamin K on BMD associated with biases and the results should be interpreted with caution (29). According to the mentioned contents, there is a controversy about the effect of vitamin K on bone regeneration and or bone formation to osteoporosis therapy. Therefore, it is necessary to study the process of differentiation of MSCs into osteoblast and mononuclear cells to osteoclasts in the presence of vitamin K2 to provide a cell therapy approach, along with osteoinduction and osteoprotective factor.

To achieve this aim, we examined the effect of vitamin K on the viability and osteogenesis potential of dental pulp stem cells, as one of the best stem cell source for bone regeneration. Moreover, the effect of this vitamin was evaluated on the differentiation of blood peripheral-mononuclear cells into osteoclast and also, their viability and function.

Materials and Methods

Ethical consideration

All protocols of the present study were approved by the ethical committee of Shahid Beheshti University of medical science. Also, tissue donors were informed about the aim of this study and written consent was obtained.

DPSCs isolation and culture

Human third molar tooth was extracted surgically from patients that underwent orthodontic treatment, without systemic and oral infection or disease, in Department of oral maxillofacial sugary of Shahid Beheshti University of Medical Sciences, Tehran, Iran. Tooth was maintained in a sterile phosphate buffer saline (PBS) solution (Sigma-Aldrich, St. Louis, Missouri, the United States), containing 5% penicillin/streptomycin (Life Technologies, California, and the United States) and 1% amphotericin B (Life Technologies, California, and the United States). Then, The Surface of the tooth was cut around the cementum-enamel junction by using sterilized dental fissure burs to reveal the pulp chamber. The pulp was immersed a digestive solution: 3mg/ml type 1 collagenase, 1% penicillin/ streptomycin (Sigma-Aldrich, St. Louis, Missouri, the United States) for 1h at 37°C. Cells were collected by centrifugation and washed twice in DMEM-high glucose (Thermo Fisher Scientific Waltham, MA, and the United States). Then, cell were seeded in a 25 cm^2 culture flask in DPSC growth medium containing DMEM-high glucose (Thermo Fisher Scientific Waltham, MA, and the United States) supplemented with 15% FBS (Thermo Fisher Scientific Waltham, MA, and the United States), 100 U/ml penicillin/ streptomycin (Life Technologies, California, and the United States), in a humidified atmosphere of 5% CO2 at 37°C. They were passaged using 0.25% trypsin/1mM EDTA (Thermo Fisher Scientific Waltham, MA, and the United States) at 90% confluence and used at third passages.

Characterization of DPSCs

The fibroblast-like cells obtained from dental pulp were harvested by treatment with 0.25% trypsin/1 Mm EDTA (Thermo Fisher Scientific Waltham, MA, and the United States) at third passage. Then, these cells incubated with 1μg per 10^6 cells FITC and PE-conjugated antibodies for 40 min at 4°C in the dark condition. FITC- conjugated antibodies were: anti-CD90 (CMG, Esfahan, IRAN), anti-CD73 (e Biosciences, San Diego, CA, and USA), anti-CD44 (EXBio, Vestec, Czech Republic), anti-CD14 (EXBio, Vestec, Czech Republic), and anti-CD45 (BD Biosciences, San

Jose, CA, USA). PE-conjugated antibodies were: anti-CD105 (EXBio, Vestec, Czech Republic) and anti-CD34 (EXBio, Vestec, Czech Republic). After washing, the analysis was performed using Attune® Acoustic Focusing Cytometer (Applied Biosystems, Foster City, CA, USA), and data analyzed using the FLOWJO 7.6.1 software (TreeStar, San Carlos, CA, USA).

Preparation and storage of vitamin K2

Vitamin K2 (Menaquinone or MK-4, V9378, Sigma-Aldrich, St. Louis, Missouri, the United States) was dissolved in absolute ethanol (Merck, Kenilworth, New Jersey, USA) at the concentrations of 5, 10 and 15 µM and. these solutions sterilized by filtration (Millipore Millex ® -GS 0.22 µm filter unit) and stored at 4°C protected from light.

Evaluation of the viability of DPSCs after exposure with vitamin K2

Cytotoxicity and cell viability were evaluated using 3-(4, 5-dimethylthiazol-2-y1)-2,5-diphenyltetrazolium bromide (MTT) assay at 1, 3, 5, 7 and 10 days at growth medium with various of the concentrations of vitamin K2. In 96 well plates, 5×10^3 cells per well were seeded in growth medium for 24 hours, then this medium was changed with growth medium containing various concentrations of vitamin K2 for 10 days.

To perform this assay, the growth medium was replaced with fresh medium containing 10% MTT solution (M5655, Sigma-Aldrich, St. Louis, Missouri, the United States) and incubated at 37°C, 5% CO_2 and protected from light. After three hours, the formazan crystals were dissolved in DMSO (Sigma-Aldrich, St. Louis, Missouri, the United States) and The optical density (OD) of each sample was recorded at 750 nm using Ultra Microplate Reader (Biotek Instruments, Winooski, VT, USA).

Evaluation of osteogenesis potential of DPSCs at exposure with vitamin K2

DPSCs were plated in 48 well plates at a confluence 3000 cell/cm2 in the growth medium. After reaching 90% confluence , the medium was changed to osteogenic medium containing DMEM-low glucose (Thermo Fisher Scientific Waltham, MA, the United States) containing 10% FBS (Thermo Fisher Scientific Waltham, MA, the United States), β-Glycerol phosphate disodium salt pentahydrate (200µg/µl) (Sigma-Aldrich, St. Louis, Missouri, the United States), acid ascorbic (10µg/µl) (Sigma-Aldrich, St. Louis, Missouri, the United States), dexamethasone (1µg/µl) (Sigma-Aldrich, St. Louis, Missouri, the United States), also, supplemented with vitamin K2 for 14 days. The effect of vitamin K2 on osteogenesis evaluated by extracellular calcium deposition staining (ECM-CDs) and gene expression using Real-time RT -PCR.

ECM-CDs staining

The ability of DPSCs to generate ECM-CDs at exposure with vitamin K2 was assessed by Alizarin Red S (ARS) staining after 14 days. For ARS staining, cells were fixed with cold 4% paraformaldehyde (PFA) (Merck, Kenilworth, New Jersey, USA) for 20 minutes and rinsed with PBS buffer and then stained with 2% Alizarin Red S (Sigma-Aldrich, St. Louis, Missouri, the United States) with PH 4.2 for 20 minutes, then cells raised by PBS buffer and observed under an inverted light microscopy (Nikon, Minato, Tokyo, Japan). The percentage of mineralization area were quantifies using Image J (free download available at http://rsbweb.nih.gov/ij/) based on five images of each group.

Evaluation of the effect of vitamin K2 on osteoclast differentiation and function

Isolation of human peripheral blood mononuclear cells (PBMNCs)

Whole blood samples from three healthy male volunteer were provided by the medical laboratory of Ayatollah Taleghani Hospital, Tehran, Iran. The Blood sample was collected into the tube containing sodium citrate dehydrate anticoagulant and then, mixed with warm PBS, equally. For isolation of mononuclear cells, This suspension was added slowly to Ficoll Paque Premium (GE Life Sciences, USA, density=1.073 g/ml) at ratio 1:1 and then the sample was centrifuged at 800g for 30 min, room temperature with minimum braking and acceleration speeds. This suspension separated into three layers, of which the small middle layer contains the PBMNCs. After washing with PBS, The cells suspended in 1 ml PBMNC growth medium containing DMEM-F12 medium (Thermo Fisher Scientific Waltham, MA, and the United States) supplemented with 10%FBS (Thermo Fisher Scientific Waltham, MA, and the United States), 10% penicillin/streptomycin (Life Technologies, California, and the United States) and 50ng ml^{-1} of M-CSF (Sigma-Aldrich, St. Louis, Missouri, and the United States). The mononuclear cells counts are performed by hand using a hemocytometer plate and light microscopic (Nikon, Minato, Tokyo, Japan) observation.

Osteoclast differentiation

PBMNCs differentiated into osteoclasts, according to the method of Marino et al. (30) with modification as follows. PBMNCs were plated into a sterile 100mm petri dish and cultured in PBMNC growth medium. After 72 hours, M-CSF -dependent macrophage (MdM) were detached gently using ice-cold PBS

and cell scraper and then 12×103 cells per well cultured in culture osteoclastogenesis medium containing DMEM-F12 medium (Thermo Fisher Scientific Waltham, MA, and the United States) supplemented with 10%FBS (Thermo Fisher Scientific Waltham, MA, and the United States), 10% penicillin/streptomycin (Life Technologies, California, and the United States) and 50ng ml^{-1} of M-CSF (M6518, Sigma-Aldrich, St. Louis, Missouri, the United States), 100 ng ml^{-1} RANKL (AB157289, ABCAM, Cambridge, the United Kingdom) and variant of concentrations of vitamin K2 (5, 10 and 15 µM) for 96 hours at 96-well plates (Figure 1).

Tartrate-resistant acid phosphatase (TRAP) enzyme staining

After 96 hours, osteoclasts were stained using TRAP staining protocols 387A**,** Sigma-Aldrich, St. Louis, Missouri, the United States), as an osteoclast differentiation marker, according to manufacturer's protocols. Then Five images per well per treatment were taken and images were quantified using Image J (free download available at http://rsbweb.nih.gov/ij/) to calculate the number of osteoclasts.

Annexin V/ Propidium Iodide (PI) assay

The percentage of apoptosis and necrosis cells was detected by Annexin V FITC Apoptosis Detection Kit (Sigma-Aldrich, St. Louis, Missouri, the United States), according to the manufacturer's guideline. Briefly, cells were detached and washed with cold PBS, then were centrifuged at 300x g for 5 min and the pellet was re-suspended and incubated in 1x binding buffer (100µl), 1 µl Annexin V-FITC and 2µl Propidium Iodide for 10 min in darkness at room temperature. The analysis was performed using flow cytometer (Applied Biosystems, Foster City, CA, USA) and data analyzed using the FLOWJO software 7.6.1 (Tree Star Software, San Carlos, California, USA).

Resorption-Pit assay

The function of osteoclasts evaluated using the resorption-pit assay (30, 31). Mature osteoclast, obtained using the methods described above, was detached and plated on slices from a Cellular Allogeneic Bone Graft (CenoBone, Tissue Regeneration Corporation, Kish, Iran) at a pH of ~6.9 osteoclastogenesis medium for 48h. Then, the slices were cleaned with a cotton swab to remove cells and stained with 1% toluidine blue in order to reveal the resorbed areas. The percentage of pit areas was reported quantitatively by measuring the ratio of pit areas to total CenoBone slices areas through five images using Image J software (free download available at http://rsbweb.nih.gov/ij/).

Real-Time RT-PCR

The osteogenic and osteoclastogenic differentiation capability of DPSCs and MdMs, respectively, was assessed by evaluation of expression osteogenic and osteoclastogenic genes using Real-Time RT-PCR. Total RNA was extracted from the cells using RNX-Plus (Sinaclon, Tehran, Iran) reagent following the manufacturer's instruction. Reverse Transcription was performed using ReverAid First Strand cDNA Synthesis kit (Sinaclon, Tehran, Iran). cDNA was subjected to Real-Time-PCR (StepOnePlus Real-Time PCR System; Applied Biosystems, Foster City, CA, USA) using a SYBR Green Master Mix Kit- high ROX (Ampliqon; Odense, Denmark). ALP, Runt-related transcription factor 1(RUNX2), Osteonectin (OCN) primers were used to assess the osteogenesis. Also, NF-κB primer was used to evaluate osteoclastogenic potential. The expression of β-actin gene was analyzed as an endogenous control. Primers used for Real-Time-PCR analysis were presented in detail in Table 1.

Statistical Analysis

All experiments were repeated three times. Quantitative data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and independent sample T-test were performed by SPSS software (Version 16.0; SPSS, Chicago, IL). The differences were considered with a confidence interval of 95% (α=0.05) for all analyses. Standard deviation error bars are showed on each figure.

Results

Expression of stem cells markers in the surface of DPSCs

Confirm the properties of stemness in isolated DPSCs, the expression of stem cell markers was evaluated using flow cytometry at third passage. This analysis showed that the DPSCs expressed a high level of stemness markers including CD90 (99.8%), CD73 (98.4%), CD44 (97.2%) and CD105 (99.5%), and also, low level of the lineage markers, such as, CD14 (1.85%), CD45 (0.53%) and CD34 (1.75%) (Figure 2).

The effect of vitamin K2 on the viability of DPSCs

The viability of DPSCs in exposure vitamin K2 increased gradually in an intra-group comparison at different days. The concentration of 5 µM of vitamin K2 showed similar results compared to the control group at all days. At the concentration of 10 µM vitamin K2, the viability increased significantly compared with other groups at different days, such that this difference has been demonstrated significantly at 10 days.

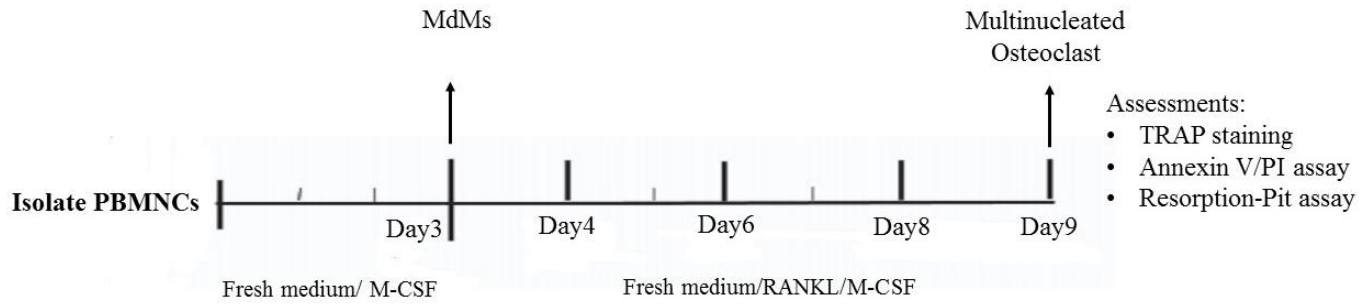
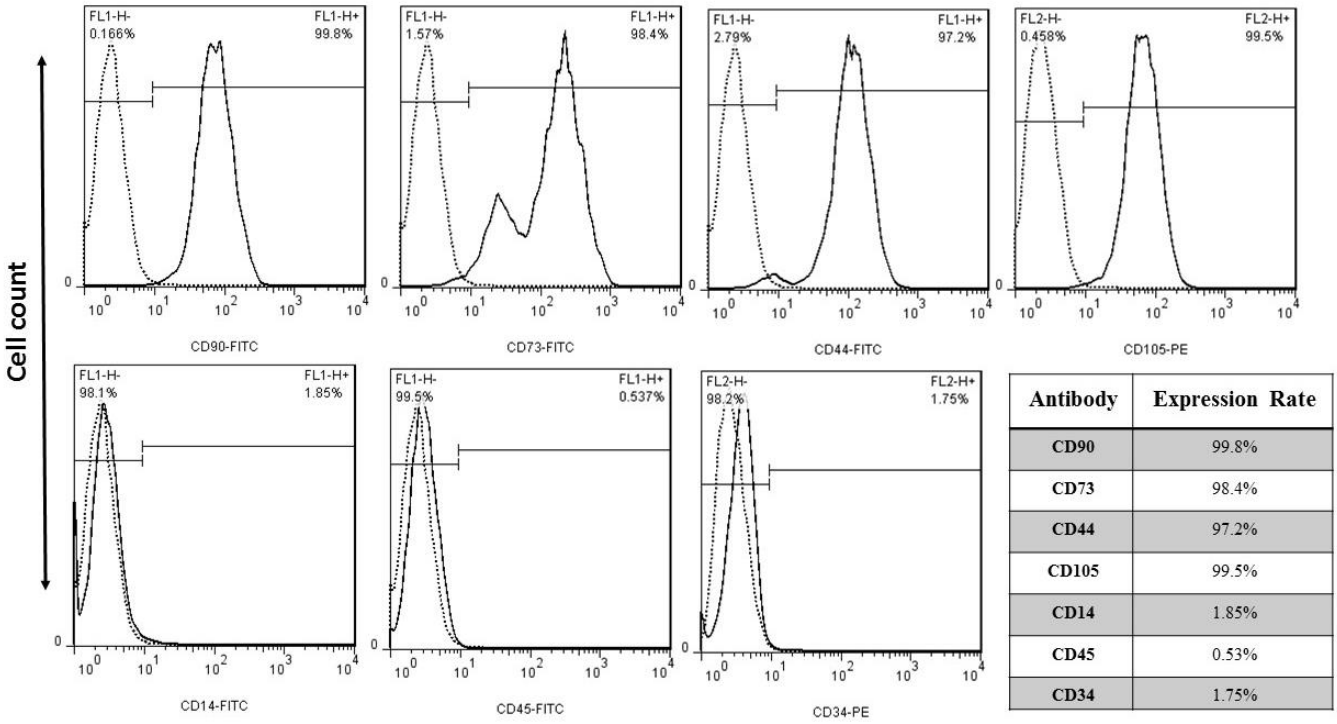


Figure 1. Flowchart of osteoclast differentiation protocol. PBMNCs: Peripheral Blood Mononuclear Cells, MdMs: M-CSF dependent Macrophages, M-CSF: Macrophage Colony-Stimulating Factor, RANKL: Receptor activator of nuclear factor kappa-B ligand, TRAP: Tartrate-resistant acid phosphatase, PI: Propidium Iodide



Antibody	Expression Rate
CD90	99.8%
CD73	98.4%
CD44	97.2%
CD105	99.5%
CD14	1.85%
CD45	0.53%
CD34	1.75%

Figure 2. Characterization of hDPSC primary cultures using flow cytometry. Histograms show fluorescence intensity and percentage Expression of surface markers. The Percentage of CD90, CD73, CD44 and CD105 were positive, while CD14, CD45 and CD34 were negative. Dash line represent the isotype control.

It is noteworthy that the potential of the viability of DPSCs decreased at 15 µM vitamin K2 than other groups. This decrease indicated significantly at 7 and 10 days (Figure 3).

The effect of vitamin K2 on the osteogenic potential of DPSCs

The effect of vitamin K2 on differentiation DPSCs into osteoblast was quantified using ARS staining, and the expression of an early osteogenic marker (ALP) and late osteogenic markers (RUNX2 and OCN) at 7 and 14 days after osteogenic induction.

ARS staining was performed to evaluate the formation of ECM-CD following osteogenic induction in the presence of vitamin K2.

ECM-DCs formed in all groups after exposure to the osteogenic medium. Moreover, quantities analysis of images indicated that the percentage of ECM-CD area was significantly higher at the concentration of 10 µM vitamin K2 than other groups (P<0.05). However, there is no significant difference between the concentrations 5 and 10 µM vitamin K2 (P=0.098) (Figure 4).

Table 1. Primers used for real time RT-PCR

Gene	Sequence 5 to 3
ALPL- F	CGGAACTCCTGACCCTTGAC
ALPL -R	ATTCTGCCTCCTTCCACCAG
RUNX2- F	GAACCCAGAAGGCACAGACA
RUNX2- R	ACTTGGTGCAGAGTTCAGGG
OCN-F	CACTCCTCGCCCTATTGGC
OCN-R	CCCTCCTGCTTGGACACAAAG
NF-κB-F	AAGGTTATTGTTCAGTTGGTC
NF-κB-R	TCTGTCATTCGTGCTTCC
B-actin-F	ATGCCTGCCGTGTGAAC
B-actin-R	ATCTTCAAACCTCCATGATG

The expression of the osteogenic genes in control group was considered as a reference point for comparison (Figure 5). The expression of ALP gene was significantly higher at the concentration of 10μM vitamin K2 at day 14th (P<0.05). However, there was no significant difference in the expression of ALP between concentrations of 5μM and 10μM vitamin K2 at day 7th. Also, no significant difference observed between 5μM and 15μM concentration of vitamin K2 at day14th. The expression of RUNX2 has been also increased significantly at osteogenic medium containing 10μM vitamin K2 at 7 and 14 days. No significant difference in the expression of this gene at the concentration of 5μM vitamin K2 between 7 and 14 days. Moreover, Quantitative gene expression analysis showed significantly increased expression of OCN in all concentrations of vitamin K2 at day 14th (P<0.05). This increase was the highest in the concentration of 10μM vitamin K2 at day14th (P<0.05). (Figure 5).

Effect of vitamin K2 on osteoclast differentiation and activity

To evaluate the effect of vitamin K2 on osteoclastogenic differentiation, PBMNCs were isolated and differentiated into osteoclasts in the presence and absence of various concentrations of vitamin K2.

The TRAP staining, as osteoclast markers, showed that the number of TRAP-positive multinucleated osteoclasts were higher at the control group compared with the vitamin K2 treated groups (P<0.05). The presence of vitamin K2 has led to a decrease in the size and the number of the cell nucleus, in a dose-dependent manner (Figure 6).

To further elucidate the mechanism of the effect of vitamin K2 on osteoclast activity, Annexin V-FITC/PI double staining was performed via flow cytometry. The percentage of apoptotic and necrotic cells reported according to LISA C. Crowley *et al.* (32). Apoptotic cells were identified from necrotic cells by co-staining

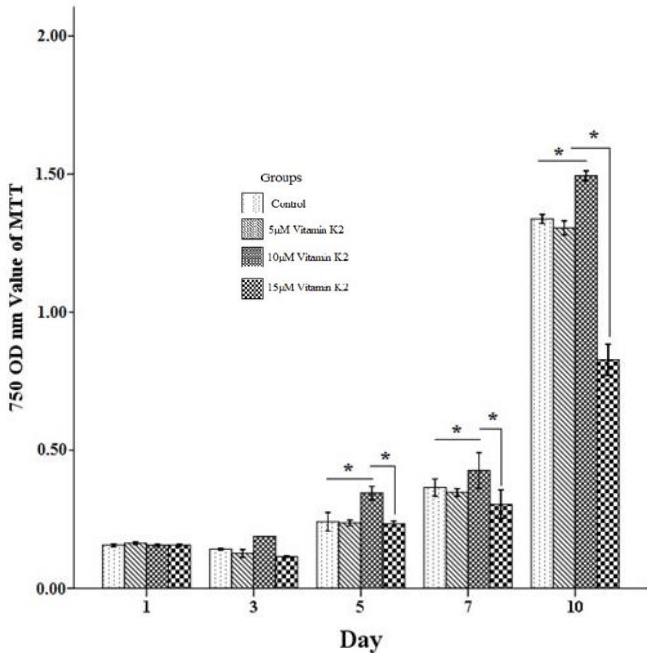


Figure 3. Formosan absorbance as a measure of cell viability measured using MTT assay. Statistical significance of the differences between the results are indicated (*P<0.05). Error bars are standard deviations (N=3)

with PI which enters necrotic cells but is deprived of apoptotic cells.

Therefore, the percentage of apoptotic cells demonstrated a significant increase in the concentrations of 10 and 15 μM compared to other groups, however, there was no difference between these groups. Moreover, the percentage of necrotic cells significantly enhanced at the concentrations of 10 and 15 μM of vitamin K2 relative to the percentage of apoptotic cells. Also, the results showed that there was a statistically significant difference in the concentration 15μM of vitamin K2 compared to all groups (Figure 7).

The expression of inflammatory factor, NF-κB, was evaluated via Real Time RT- PCR. The expression of NF-κB was significantly lower at differentiated osteoclast in the presence of various concentrations of vitamin K2 compared to the control group at 7 days. Also, No significant difference observed in various concentrations of Vitamin K2 (Figure 8).

To the confirmation of functionality of mature osteoclast phenotype, Resorption-pit assay was conducted in differentiated osteoclasts in presence and absence of vitamin K2. It was clear that the area of pit assay was significantly reduced by the presence of vitamin K2, in a dose-dependent manner. No significant difference was observed between 10 and 15 μM vitamin K2 concentrations (Figure 9).

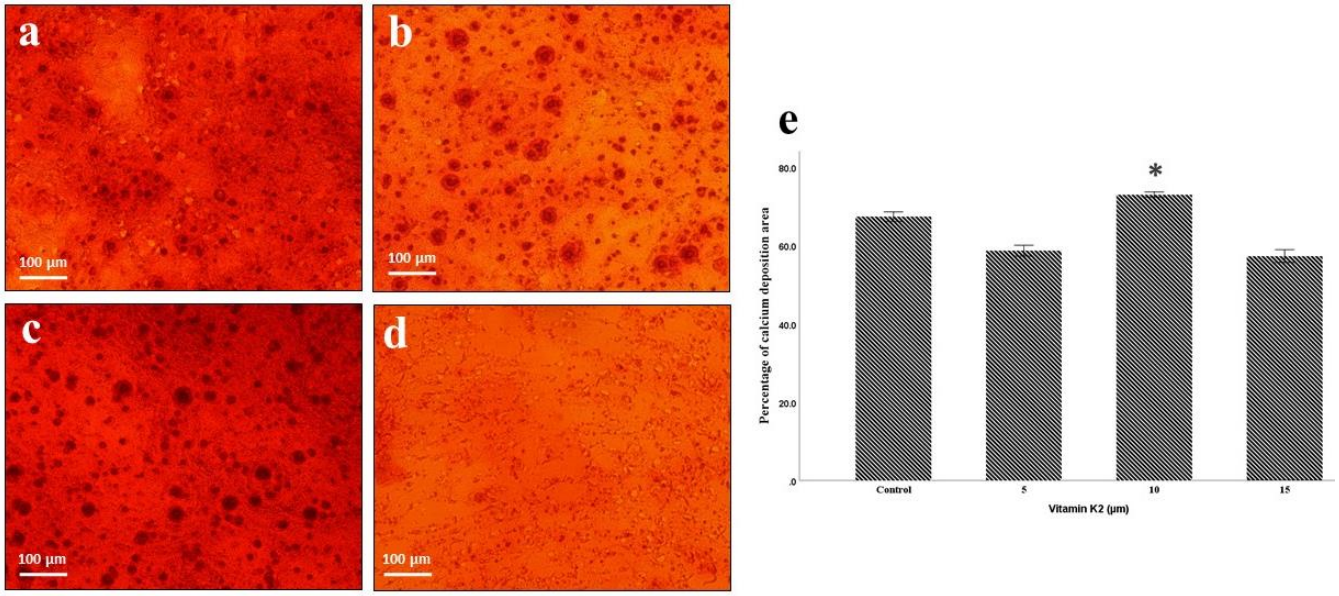


Figure 4. The extracellular matrix- calcium deposition (ECM-DCs) was stained by ARS. a) DPSC were cultures in osteogenic medium at 14 day. b, c, d) DPSC were cultured in osteogenic medium with the concentration of 5, 10 and 15µM vitamin K2 after 14 days, respectively. e) ARS staining was quantifies by measuring the area of coloration using ImageJ software. Statistical significance of the differences between the results are indicated (* *P*<0.05). Error bars are standard deviations (N=3)

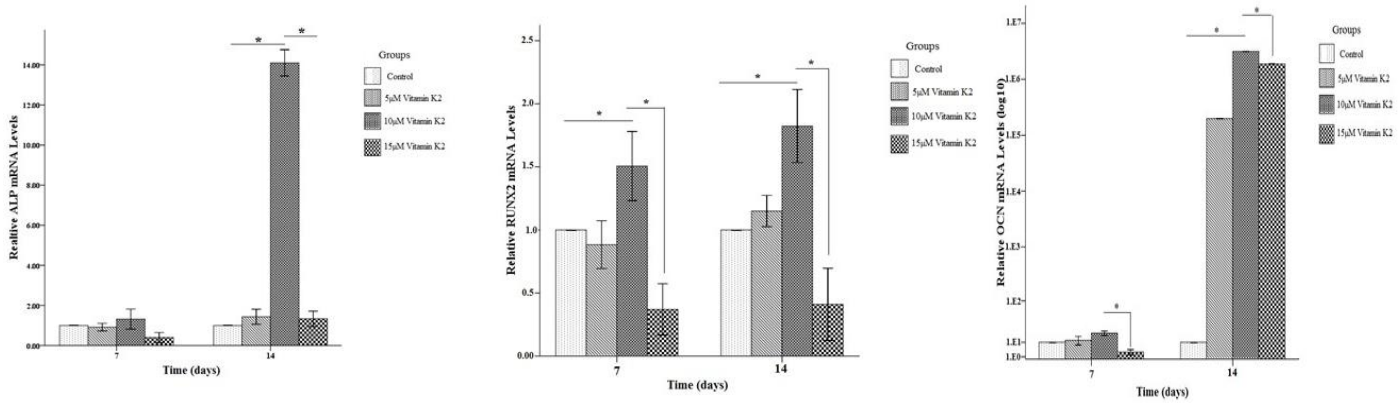


Figure 5. Relative gene expression measured using Real-Time RT-PCR. Statistical significance of the differences between the results are indicated (*P<0.05). Error bars are standard deviations. (N=3) .ALP: Alkaline Phosphatase, RUNX2: Runt-Related Transcription Factor 2 and OCN: Osteocalcin

Discussion

According to the international osteoporosis foundation report about the prevalence of osteoporosis with the aging of people in the world, achieving a therapeutic approach to bone regeneration and repair is necessary rather than antiresorptive therapies (1, 2, 5, 8).

In recent years, stem cell therapy introduces as the goal for osteoporosis therapy, because these cells have differentiation potential to osteoblast and enhance the bone formation process (1, 2, 9, 12, 13). addition to, Mesenchymal stem cells have immunosuppressive potential and also the use of these cells is not associated with concern about transformation and immune rejection as well as easily isolated from various sources such as bone marrow, adipose tissue, and dental pulp (7, 14, 33).

From the perspective of pathophysiology, this disease caused by misbalance between bone formation and bone resorption and also, The stem cells of these patients tend to be more differentiated into adipocyte (4, 5, 7). Therefore, Choose a suitable source of stem cells is of great importance.

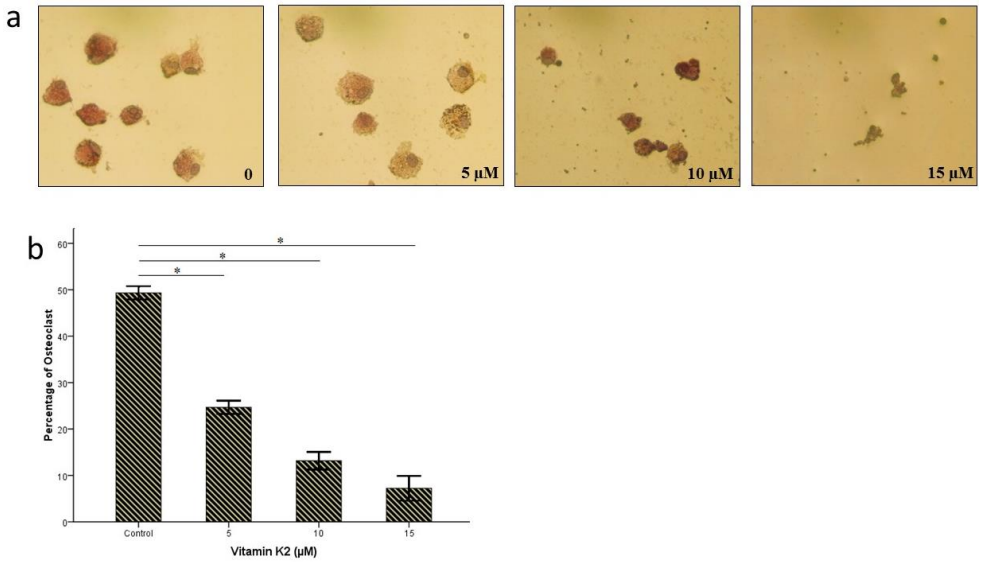


Figure 6. TRAP staining. A) Representative pictures of TRAP staining and b) Quantification of percentage of TRAP positive osteoclasts in presence and absence of various concentration of vitamin K2. Statistical significance of the differences between the results are indicated (* P<0.05). Error bars are standard deviations. (N=3). TRAP: Tartrate-Resistant Acid Phosphatase

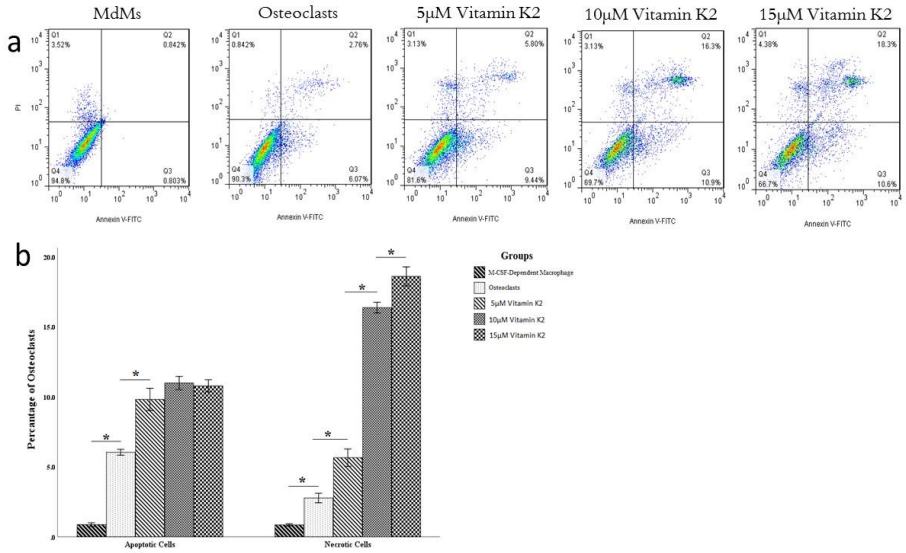


Figure 7. Evaluation of apoptosis and necrosis by Annexin V-FITC/PI staining and analysis by flow cytometry. a) The dot plot diagrams represent in the different groups. b) Quantification of percentage of apoptosis and necrosis osteoclasts. Statistical significance of the differences between the results are indicated (* P<0.05). Error bars are standard deviations. (N=3). MdMs; M-CSF-dependent Macrophages FITC, fluorescein isothiocyanate; PI, propidium iodide

In this study, we chose dental pulp stem cells for various reasons: 1) the isolation of these cells from, as the autologous source, is much easier than bone marrow tissue (34, 35), and 2) have less tendency to differentiate into adipocyte compared to fat tissue-derived stem cells(36). Moreover, Kim et al and colleagues used from tonsil-derived mesenchymal stem cell to the evaluation of safe and effective stem cell therapy for osteoporosis. This study showed that use of TMSC failed to reverse osteoporosis (5).

The several studies performed to enhance homing of MSCs and their viability and also differentiation into osteoblast or bone formation. In order to achieve these objectives, gene and surface protein expression of mesenchymal stem cells modified genetically through viral and non-viral vectors (5, 7, 16-18). But to date, none of these methods have been used in clinical practice.

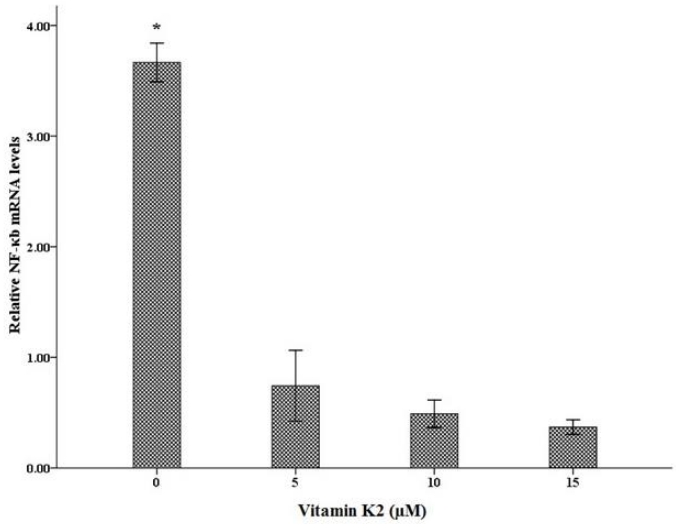


Figure 8. Relative NF-κB gene expression measured using Real-Time RT-PCR. Statistical significance of the differences between the results are indicated (* P<0.05) (N=3) .NF-κB: Nuclear Factor Kappa-B

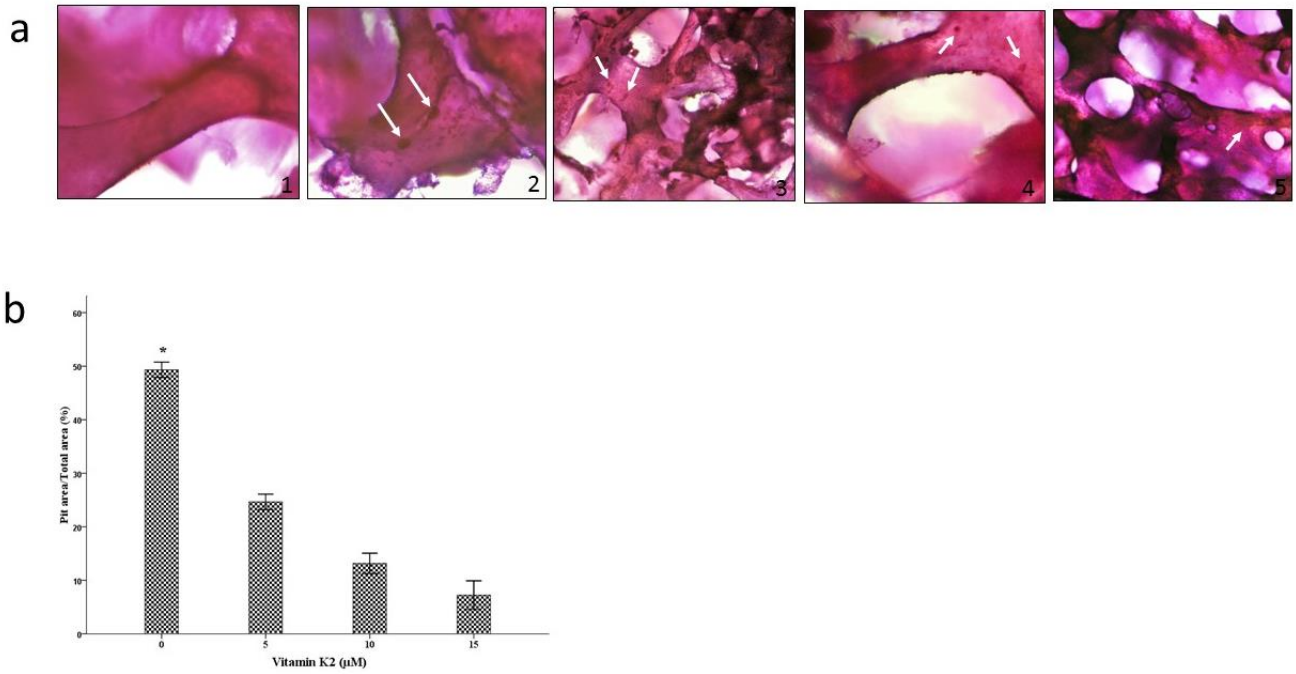


Figure 9. Evaluation of pit formation by Toluidine blue staining and analysis by invert microscopy. a) Representative pictures of Toluidine blue staining; 1) M-CSF Dependent Macrophage; 2, 3, 4, 5) differentiated osteoclast with 0, 5, 10 and 15µM vitamin K2, respectively. b) Quantification of percentage of pit area to total area was measured using ImajeJ software. Statistical significance of the differences between the results are indicated (* P<0.05) (N=3)

Since the 1990s, the family of vitamin K, especially vitamin K2 or MK-4, is known as a medicine to reduce the symptoms of the osteoporosis, as an osteoprotective factor, and also the several in vitro study demonstrated that vitamin K2 promotes osteogenesis and inhibit osteoclastogenesis (25-28, 37). Nonetheless, a meta-analysis study to evaluate the effect of vitamin K in the clinical trials reported that this vitamin has no significant effect in the reduction the osteoporosis symptoms (29). One of the reasons for this challenge may be the use from osteoblast and osteoclast cell line and or stem cells of animal origin that these cells are different biologically and physiologically with normal human cells.

Therefore, our aim in this study was to investigate the effect of vitamin K2 (MK-4) and select the best dose to increase viability and osteoblast differentiation potential in dental pulp-derived stem cells as a cell source to osteoporosis therapy, then the effect of this vitamin on differentiation and activity of peripheral mononuclear cells derived osteoclast evaluated. The several concentrations of vitamin K2 (5, 10 and 15 μM) selected by evaluation of previous studies.

Our finding showed that MK4 at the concentration of 10 μM enhances the viability and proliferation of dental pulp stem cells in growth medium condition. This results showed that this dose can support and lead to increased vitality of stem cells before their differentiation into osteoblast in vivo condition.

The evaluation of osteogenesis markers such as ECM-DCs formation showed that DPSCs have significant potential of osteogenic differentiation in the presence of the concentration of 10 μM of vitamin K2 at osteogenic medium. Osteogenic gene expression analysis also observed that the concentration of 10 μM of vitamin K2 significantly improved the expression of ALP, RUNX2, and OCN gene. In addition, ALP activity, as an early osteogenesis differentiation markers, assessed by Rasouli-Ghahroudi and coworkers. They applied that the concentration of 10 μM of vitamin K 2 dramatically increased differentiation DPSCs into osteoblasts after 14 days (38). In agreement with these findings, other study indicated the significant increase in the percentage of ALP+ subpopulation, as the indicative pre-osteoblast cells, in human bone marrow culture, when 10 μM MK4 was administrated(26). In addition, several studies showed that vitamin K2 induces the expression of osteogenic genes, especially OCN gene, through xenobiotic receptor (SXR)/ preganane X receptor (PXR) pathway (25, 39).

On the other hand, the previous studies suggested that vitamin K2 inhibits the osteoclast formation and proliferation and it doesn't lead to a decrease in cell viability or an increase in apoptosis. Moreover, they argued that this vitamin has any cytotoxic effect for bone marrow stromal cells (35.25) However, the present finding demonstrated morphological signs of cell death, such as shrinkage of the cells, formation of apoptotic bodies, and condensation of chromatin around the nucleus, in the osteoclast after TRAP staining, and also quantities analysis approved a dose-dependently decrease in TRAP-positive cells. For future evaluation, we estimated the percentage of apoptotic cells using Annexin V/ PI assay that this indicated vitamin K2 significantly enhanced the apoptosis osteoclasts compared to control groups after differentiation process, although, the enhancement was no significant between the various concentrations of vitamin K2. In addition, the study showed a dramatically and dose-dependently increase in the percentage of necrotic osteoclasts after differentiation along with vitamin K2. The previous studies approved that the necrosis process is associated with widespread tissue damage and an intense inflammatory response through the ultimate breakdown of the plasma membrane and the release lysosomal enzymes into the extracellular matrix (40, 41). Therefore, our finding suggested that a high concentration of vitamin K2 delay tissue regeneration due to the promotion of necrosis and tissue inflammation.

Osteoclastogenesis is assumed to be controlled by the balance between NF-κB and OPG expression in vivo condition. Several studies proved that a decrease in osteoclast differentiation happens as a result of reducing NF-κB expression (26, 42). However, others showed that osteoclast activity reduced due to increasing OPG expression produced by osteoblasts and block the NF-κB receptor (43, 44). Koshihara and co-workers suggested that at higher concentration of 10μM inhibited 1, 25 dihydroxyvitamin D3-induced osteoclast formation through reduction of NF-κB and overexpression of OPG expression. Our findings were consistent with the previous studies so that the expression of NF-κB significantly dropped in PBMNCs derived osteoclasts a dose-dependent manner.

Despite the previous studies reported that vitamin K2 decreased osteoclasts by changing the mechanism of differentiation or proliferation, but have not shown the effect of this vitamin on the osteoclast function and their osteoporosis mechanism. In vitro osteoclast- mediated bone resorption can be evaluated using resorption-pit assay. Therefore, in the present study, osteoporosis function of osteoclasts was assessed through pit formation assay after osteoclast differentiation in the presence of vitamin K2. Hence, the statistical analysis showed vitamin K2 noticeably suppressed the number osteoclast-mediated formation of the resorption pit.

Conclusion:

It can be concluded that vitamin K2 has an important dual role in the promotion of osteogenesis and inhibition of osteoporosis at various concentrations. Among these concentrations of vitamin K2, our finding demonstrated the concentration of 10 μM of vitamin K2 presents the best efficacy on the promotion of bone regeneration. In view of the fact that cell viability and osteogenic

potential of DPSCs noticeably increased in this concentration than other groups. On the other hand, the 10 µM vitamin K2 presented an acceptable level of inhibitory of osteoclastogenesis and osteoclast function. As a result, knowing the mechanism involved in the dual functionality of vitamin K2, the concentration of 10uM would be recommended for the purpose of stem cell therapy for bone regeneration in future in vivo studies.

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