The Effect of Vitamin K2 on Osteogenic Differentiation of Dental Pulp Stem Cells: An In Vitro Study

Amir Alireza Rasouli-Ghahroudi [a], Solmaz Akbari [b], Mohammad Najafi-Alishah [c], Mahbobeh Bohloli [d*]

[a] Periodontics Department and Dental Implant Research Center, Dental School, Tehran University of Medical Sciences, Tehran, Iran; [b] Periodontics Department, Dental School, Tehran University of Medical Sciences, Tehran, Iran; [c] Dental School, Tehran University of Medical Sciences, Tehran Iran; [d] Department of Tissue Engineering and Applied Cell Sciences, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran

Corresponding author:** Mahbobeh Bohloli, Department of Tissue Engineering and Applied Cell Sciences, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran. ***E-mail:mahbobeh.bohloli@yahoo.com, ***Tel:*** +98 21 22439848

Submitted: 2017-08-01; **Accepted:** 2017-08-14

Introduction: dental pulp stem cells (DPSCs) have been shown to have great capacity to differentiation toward the osteoblast lineage and they can be considered as a great cell source for bone tissue engineering. The vitamin K family, especially vitamin K2 (MK-4), have been shown to have an osteoprotective role. In this study, we have investigated the effect of various concentrations of MK-4 on differentiation of DPSCs into osteoblast. **Materials and Methods:** DPSCs were isolated and characterized to expression the mesenchymal markers. These cells were treated with osteogenic medium with and without of various concentrations of MK-4 for 14 days. Osteogenic capability and extracellular calcium deposition were assessed by ALP assay and alizarin red staining, respectively, at zero, 7, 14 days after induction. **Result:** the additional of MK-4 at concentration of 10 µM with osteogenic medium had a significant effect on differentiation DPSCs into osteoblast ($P<0.05$) at 14 day, as it confirmed by both ALP activity assay and alizarin red staining. **Conclusion:** MK-4 can promote differentiation of DPSCs into osteoblast in vitro so have a potential to be considered in improvement of cell-based bone tissue engineering therapies.

Keyword: Mesenchymal stem cells; Dental pulp stem cells; Vitamin K; Osteogenesis

Introduction

Dental pulp stem cells (DPSCs) are the mesenchymal stem cells (MSC) population present in adult teeth, showing similar features to the MSCs derived from bone marrow and adipocyte tissue (1). These cells have the capacity for self-renewal and the potential for differentiation into multiple cell lineages, especially osteoblasts (1-5). Also, the isolation procedure of DPSCs from tooth tissues is relatively non-invasive process(6). Previous studies have emphasized that DPSCs represent an excellent source for bone regeneration (3, 4, 7).

To date, the attention of many researchers has been focused to enhance the osteogenic capability of MSCs for the purpose of the bone tissue engineering. Previous studies investigated the effect of various osteoinductive factors, including bone morphogenetic proteins (BMPs) , dentin matrix proteins, transforming growth factor beta(TGF-β), stromal cell-derived factor 1 (SDF-1), platelet-derived growth factor (PDGF) and purmorphamine, in order to improve the osteogenic capability of MSCs(2, 8-12).

Several studies have been shown that the Vitamin K as group of fat-soluble vitamins, is essential for blood coagulation as well as bone formation and remodeling in mammals (13-17). Vitamin K naturally occurs in two forms: phyloquinones (K1) and menaquinines (K2) (18-20). The most common form of K2, menaquinine 4 (MK4), has a greater osteoprotective effect (19). Studies have been demonstrated that MK4 has great importance in inhibition of prostaglandin E2 synthesis, apoptotic cell death of osteoblast and also enhances human osteoblast-induced mineralization with or without coincubation with 1, 25-hydroxyvitamin D_3 [1, $25(OH)_2D_3$] and maintain bone strength via the ɤ-carboxylation of osteocalcin(13, 16, 18, 21, 22).

In the present study, we tend to answers the question that whether MK4 stimulates differentiation of DPSC to osteoblast in vitro.

Materials and Methods

Dulbecco's modified Eagle's medium (DMEM), Fetal Bovine Serum (FBS), Penicillin Streptomycin (pen/strep), Amphotericin B and Trypsin/EDTA were purchased from (Life Technologies, California, United States, and Cell Lysis Reagent (C3228), Alkaline Phosphatase Yellow (pNPP) Liquid Substrate (P7998), Alizarin Red (A3757), DAPI, β-Glycerol

phosphate disodium salt pentahydrate, acid ascorbic and dexamethasone from Sigma-Aldrich, St. Louis, Missouri, United States.

Human dental pulp extraction and cell culture

Human dental pulp were isolated from third molar of healthy volunteer adult donors without systemic and oral infection or diseases. The inform consent forms were obtained from patients. Tooth were placed in sterile phosphate buffered saline (PBS) containing 5% pen strep. Dental pulp was obtained with a dentinal excavator or a Gracey curette. The pulp was immersed in a digestive solution composed of 3mg/ml type I collagenase at 37° C for 60 minutes. Standard medium (DMEM-high glucose (HG) with 15% FBS was added to the cells before the suspension was centrifuged at room temperature (RT) for 5 minutes at 300g. The cell pellet was suspended in DMEM-HG with 15% FBS and 1% pen strep and placed in 25 cm^2 flasks. Cells were maintained in a humidified atmosphere under 5% CO_2 at 37° C and media were changed twice a week. When DPSC reached 70% confluence, they were detached using 0.25% trypsin/1mM EDTA.

Characterization of DPSCs

DPSCs were characterized for the expression of MSCs markers, CD73, CD90, CD105 and CD44, and the weak expression for CD34 and CD45. At P3, DPSCs were treated with 0.05% trypsin-EDTA. The harvested cells are centrifuged and then, cell pellet was re-suspended at concentration of 10^6 per sample in PBS and incubated for 30 min at room temperature in the dark with antibodies. After incubation time, cells were washed with PBS. Analysis was performed using flow cytometer.

Osteogenic differentiation induction

Cells were incubated triplicate in wells of a 24- well plate (5×10^3 cells/well) in osteogenic medium (DMEM-Low Glucose containing 10% FBS, β-Glycerol phosphate disodium salt pentahydrate (200μg/μl), acid ascorbic (10μg/μl),dexamethasone (1μg/μl), 1% pen strep antibiotic) in the presence or absence of various concentrations of MK-4 (15, 10, 5 μM) for 14 day. Medium were replaced at twice a week. Cells were evaluated for osteogenic capability and the extracellular calcium deposition by alkaline phosphatase (ALP) activity assay and alizarin red staining, respectively, after 7 and 14 day induction.

Quantitative ALP activity assay

For ALP activity assay, cells were rinsed with PBS buffer and homogenized in Cell Lysis Reagent for 30 min with gentle agitation. The resulting mixture in then centrifuged at 20.000g for 20 min at 4°C. The cell lysate was mixed with Alkaline Phosphatase Yellow (pNPP) Liquid Substrate solution, after incubation at room temperature for 30 min, the above mixture is added to 1 N NaOH to stop the reaction and the absorbance at 405 nm was measured using ELISA reader.

Alizarin Red staining

For staining, the cell culture were fixed in cold 4% paraformaldehyde (PFA) for 20 minutes and rinsed with PBS buffer cells were stained with 2% alizarin red solution buffered at a pH of 4.2 for 20 minutes, then cells were rinsed by PBS buffer and observed under an inverted microscopy.

Statistical analysis

The results were obtained from the triplicate samples. Data were expressed as mean ±SD. Statistical analysis was carried out using SPSS V16.0 software by one-way ANOVA followed by the Tukey's multiple comparison test. $P<0.05$ was considered statistically significant.

Results

MSCs phenotype of DPSCs

To ensure that the DPSCs have minimal criteria to be called MSCs. The DPSCs were analyzed by flow cytometry to monitor the surface marker profile. These cells showed high expression level of markers, CD44, CD73, CD90, and CD105. As expected, CD34 and CD45 detected on low expression level (**Error! Reference source not found.**).

Effect of MK-4 on ALP activity

ALP activity assay was used as an early maker of osteogenic differentiation. We compared ALP activity level between DPSCs induced to osteoblast with and without the presence of various concentrations of MK-4 (15, 10, 5 μM) at 0, 7 and 14 day. There was a statistically significant change in ALP activity level in all groups at 14 day as it compared to 7day. These results showed that cells induced by osteogenic medium with 10μM MK-4 had a high level of ALP activity compared to other groups.

Effect of MK-4 on extracellular calcium deposition

The extracellular calcium deposition, as a marker of the late stage of osteogenesis, was stained by alizarin red staining. All group demonstrated a high level of calcium deposition relative to 7day. Also significantly, osteogenic medium with 10μM MK-4 had high osteogenic efficacy at 14 day compared to other groups.

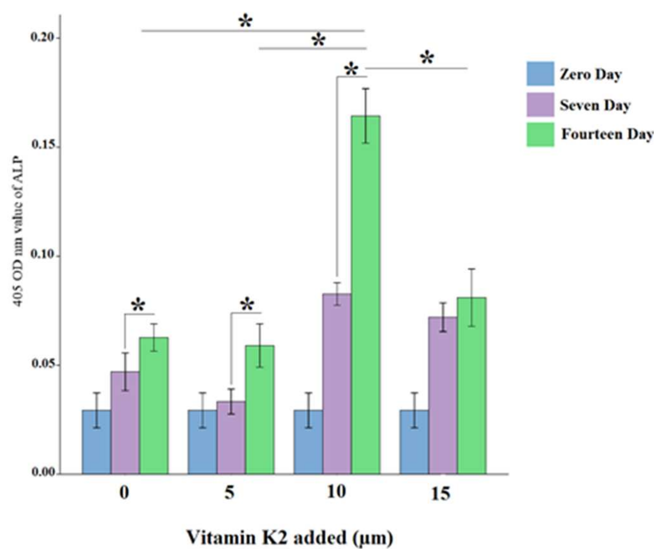

Figure 1. Alkaline Phosphatase (ALP) activity was measured using the p-nitrophenyl phosphate assay. Statistical significance of the differences between the results are indicated (* *P*<0.05)

Table 1. DPSCs surface marker expression, assessed by flow cytometry analysis

Antibody	Expression Rate (%)
CD90	99.7
CD73	98
CD44	97.5
CD105	99
CD34	1.5
CD45	0.5

Discussion

Previous studies have reported that enhancement of osteogenesis of MSCs in vitro is a vital factor in bone tissue engineering (23). DPSCs with a great potential to differentiation toward the osteoblast have been to have a capacity to use for bone tissue engineering in clinical setting (1, 3, 5). Some studies have indicated that MK-4 intake accelerates bone loss in human and animal models (24-27), also in vitro studies have confirmed a role of MK-4 as an osteoprotective factor (16, 18-20). In the current study, we evaluated the osteoinductive effect of MK-4 on differentiation DPSCs to osteoblast in vitro. Results of ALP activity and calcium deposition have been shown that MK-4 can enhancement of osteoblast differentiation.

In present study, we evaluated the effect of various concentrations of MK-4 (5, 10, 15 µM) on differentiation DPSCs into osteoblast for 14 day in vitro. ALP assay and alizarin red staining have shown that 10 µM concentration of vitamin K2, significantly increased differentiation DPSC to osteoblast in compared to other groups. Similar to our observation, Igarashi et al. reported that 10µM of MK4 promoted gene expression of Msx2, as osteoblastogenic factors, in osteoblast cell line (MC3T3-E1) (19). Y Koshihara *et al*. evaluated the effect of different concentration of vitamin K1 and K2 from 1.0 µM to 10µM on osteoblastogenesis and osteoclastogenesis and they also suggested that 10µM of MK-4 can induce the differentiation of mononuclear cells isolated from human bone marrow to osteoblast. Also, they showed that this concentration decreased osteoclast formation from bone marrow cells by an increase in cell death(20). Atkins et al showed that 10µM of MK-4 promotes mineralization, osteoblast-to-osteocyte transition on MLO-Y4 cells(18). In this study, we evaluated the osteogenic effect of MK-4 on MSCs derived from dental pulp. In contrast, all other reported studies have conducted on osteoblast cell line. However, cell lines are not proper for evaluation of osteoinduction since, they are mature and terminally differentiation.

The above mentioned studies confirm our results for the effect of MK-4 on differentiation into osteoblast. Nevertheless, further studies are needed to investigating the effect of MK-4 on gene expression involved in differentiation into osteoblast and evaluation of differentiation peripheral blood mononuclear cell into osteoclast, as the main factor in bone tissue regeneration.

Conclusion

This study shows that MK-4 can promote differentiation of DPSCs towards the osteoblast in vitro, hence it can be suggested to be used in cell-based bone tissue engineering therapies for bone regeneration enhancement.

Conflict of Interest: 'None declared'.

References

1. Mori G, Brunetti G, Oranger A, Carbone C, Ballini A, Muzio LL, et al. Dental pulp stem cells: osteogenic differentiation and gene expression. Annals of the new York Academy of Sciences. 2011;1237(1):47-52.
2. Aurrekoetxea M, Garcia-Gallastegui P, Irastorza I, Luzuriaga J, Uribe-Etxebarria V, Unda F, et al. Dental pulp stem cells as a multifaceted tool for bioengineering and the regeneration of craniomaxillofacial tissues. Frontiers in physiology. 2015;6.
3. Di Benedetto A, Brunetti G, Posa F, Ballini A, Grassi FR, Colaianni G, et al. Osteogenic differentiation of mesenchymal stem cells from dental bud: role of integrins and cadherins. Stem cell research. 2015;15(3):618-28.

4. Ledesma-Martínez E, Mendoza-Núñez VM, Santiago-Osorio E. Mesenchymal stem cells derived from dental pulp: a review. Stem cells international. 2015;2016.
5. Morad G, Kheiri L, Khojasteh A. Dental pulp stem cells for in vivo bone regeneration: a systematic review of literature. Archives of oral biology. 2013;58(12):1818-27.
6. Potdar PD, Jethmalani YD. Human dental pulp stem cells: applications in future regenerative medicine. World journal of stem cells. 2015;7(5):839.
7. Khojasteh A, Nazeman P, Rad MR. Dental stem cells in oral, maxillofacial and craniofacial regeneration. Dental Stem Cells: Springer; 2016. p. 143-65.
8. Behnia H, Khojasteh A, Esmaeelinejad M, Naghdi N. Effects of different growth factors on new bone formation: A systematic review. Journal of Islamic Dental Association of IRAN (JIDAI). 2012;24(2):2.
9. Behnia H, Khojasteh A, Soleimani M, Tehranchi A, Atashi A. Repair of alveolar cleft defect with mesenchymal stem cells and platelet derived growth factors: a preliminary report. Journal of Cranio-Maxillofacial Surgery. 2012;40(1):2-7.
10. Rad MR, Khojaste M, Shahriari MH, Asgary S, Khojasteh A. Purmorphamine increased adhesion, proliferation and expression of osteoblast phenotype markers of human dental pulp stem cells cultured on beta-tricalcium phosphate. Biomedicine & Pharmacotherapy. 2016;82:432-8.
11. Rad MR, Liu D, He H, Brooks H, Xiao M, Wise GE, et al. The role of dentin matrix protein 1 (DMP1) in regulation of osteogenic differentiation of rat dental follicle stem cells (DFSCs). Archives of oral biology. 2015;60(4):546-56.
12. Yao S, He H, Gutierrez DL, Rad MR, Liu D, Li C, et al. Expression of bone morphogenetic protein-6 in dental follicle stem cells and its effect on osteogenic differentiation. Cells Tissues Organs. 2013;198(6):438-47.
13. Koshihara Y, Hoshi K, Ishibashi H, Shiraki M. Vitamin K 2 promotes 1α, 25 (OH) 2 vitamin D 3-induced mineralization in human periosteal osteoblasts. Calcified tissue international. 1996;59(6):466-73.
14. Shiraki M, Shiraki Y, Aoki C, Miura M. Vitamin K2 (menatetrenone) effectively prevents fractures and sustains lumbar bone mineral density in osteoporosis. Journal of bone and mineral research. 2000;15(3):515-21.
15. Takeuchi Y, Suzawa M, Fukumoto S, Fujita T. Vitamin K 2 inhibits adipogenesis, osteoclastogenesis, and ODF/RANK ligand expression in murine bone marrow cell cultures. Bone. 2000;27(6):769-76.
16. Urayama S, Kawakami A, Nakashima T, Tsuboi M, Yamasaki S, Hida A, et al. Effect of vitamin K 2 on osteoblast apoptosis: Vitamin K 2 inhibits apoptotic cell death of human osteoblasts induced by Fas, proteasome inhibitor, etoposide, and staurosporine. Journal of Laboratory and Clinical Medicine. 2000;136(3):181-93.
17. Vermeer C, Jie K-S, Knapen M. Role of vitamin K in bone metabolism. Annual review of nutrition. 1995;15(1):1-21.
18. Atkins GJ, Welldon KJ, Wijenayaka AR, Bonewald LF, Findlay DM. Vitamin K promotes mineralization, osteoblast-to-osteocyte transition, and an anticatabolic phenotype by γ-carboxylation-dependent and-independent mechanisms. American Journal of Physiology-Cell Physiology. 2009;297(6):C1358-C67.
19. Igarashi M, Yogiashi Y, Mihara M, Takada I, Kitagawa H, Kato S. Vitamin K induces osteoblast differentiation through pregnane X receptor-mediated transcriptional control of the Msx2 gene. Molecular and cellular biology. 2007;27(22):7947-54.
20. Koshihara Y, Hoshi K, Okawara R, Ishibashi H, Yamamoto S. Vitamin K stimulates osteoblastogenesis and inhibits osteoclastogenesis in human bone marrow cell culture. Journal of Endocrinology. 2003;176(3):339-48.
21. Hara K, Akiyama Y, Nakamura T, Murota S, Morita I. The inhibitory effect of vitamin K2 (menatetrenone) on bone resorption may be related to its side chain. Bone. 1995;16(2):179-84.
22. Tsugawa N, Shiraki M, Suhara Y, Kamao M, Tanaka K, Okano T. Vitamin K status of healthy Japanese women: age-related vitamin K requirement for γ-carboxylation of osteocalcin. The American journal of clinical nutrition. 2006;83(2):380-6.
23. Houshmand B, Behnia H, Khoshzaban A, Morad G, Behrouzi G, Dashti SG, et al. Osteoblastic differentiation of human stem cells derived from bone marrow and periodontal ligament under the effect of enamel matrix derivative and transforming growth factor-beta. Oral & Craniofacial Tissue Engineering. 2011;1(3).
24. Fang Y, Hu C, Tao X, Wan Y, Tao F. Effect of vitamin K on bone mineral density: a meta-analysis of randomized controlled trials. Journal of bone and mineral metabolism. 2012;30(1):60-8.
25. Knapen M, Schurgers L, Vermeer C. Vitamin K2 supplementation improves hip bone geometry and bone strength indices in postmenopausal women. Osteoporosis International. 2007;18(7):963-72.
26. Ochiai M, Nakashima A, Takasugi N, Kiribayashi K, Kawai T, Usui K, et al. Vitamin K2 alters bone metabolism markers in hemodialysis patients with a low serum parathyroid hormone level. Nephron Clinical Practice. 2011;117(1):c15-c9.
27. Sogabe N, Maruyama R, Baba O, Hosoi T, Goseki-Sone M. Effects of long-term vitamin K 1 (phylloquinone) or vitamin K 2 (menaquinone-4) supplementation on body composition and serum parameters in rats. Bone. 2011;48(5):1036-42.

Please cite this paper as: Rasouli-Ghahroudi AA, Akbari S, Najafi-Alishah M, Bohloli M. The Effect of Vitamin K2 on Osteogenic Differentiation of Dental Pulp Stem Cells: An In Vitro Study. Regen Reconstr Restor. 2017;2(1):26-29.