

Original Article

The Effect of 1 α ,25(OH)2D3 on Osteogenic Differentiation of Stem Cells from Dental Pulp of Exfoliated Deciduous Teeth

Farzad Mojarad ¹, Iraj Amiri ², Rezvan Rafatjou ¹, Atousa Janeshin ³, Maryam Farhadian ⁴

¹ Dept. of Pediatric Dentistry, School of Dentistry, Hamadan University of Medical Science, Hamadan, Iran.

² Dept. of Anatomy, Hamadan University of Medical Science, Hamadan, Iran.

³ Dept. of Pediatric Dentistry, School of Dentistry, Guilan University of Medical Science, Rasht, Iran.

⁴ Dept. of Biostatistics, Modeling of Noncommunicable Diseases Research Center, Department of Biostatistics, School of Public Health, Hamadan University of Medical Sciences, Hamadan, Iran.

KEY WORDS

Stem Cells;
Dental Pulp;
Deciduous Tooth;
1 α ,25-dihydroxyvitamin D3;

ABSTRACT

Statement of the Problem: Stem cells from human exfoliated deciduous teeth (SHEDs) are a population of highly proliferative cells, being capable of differentiating into osteogenic, odontogenic, adipocytes, and neural cells. Vitamin D3 metabolites such as 1 α , 25-dihydroxyvitamin D3 are key factors in the regulation of bone metabolism.

Purpose: The aim of this study was to investigate the effect of 1 α , 25-dihydroxyvitamin D3 on osteogenic differentiation (alkaline phosphatase activity and alizarin red staining) of stem cells of exfoliated deciduous teeth.

Materials and Method: Dental pulp was removed from freshly extracted primary teeth and immersed in a digestive solution. Then, the dental pulp cells were immersed in α -MEM (minimum essential medium) to which 10% fetal bovine serum was added. After the third passage, the cells were isolated from the culture plate and were used for osteogenic differentiation. As a control group, the cells were cultured in osteogenic cell culture medium. As the case group, the cells were cultured in osteogenic culture medium supplemented with 100 nM 1 α ,25(OH)2D3. The alkaline phosphatase (ALP) activity and alizarin red staining were analyzed to evaluate the osteogenic differentiation at day 21. The results were analyzed by using t-test.

Results: Compared with the control group, significant increase was observed in ALP activity of SHEDs after being treated with 1 α ,25(OH)2D3 ($p=0.002$). Alizarin red staining demonstrated that the cells exposed to 1 α ,25(OH)2D3 induced higher mineralized nodules ($p<0.001$).

Conclusion: Osteoblast differentiation in SHEDs was stimulated by 1 α ,25(OH)2D3. It can be concluded that 1 α ,25(OH)2D3 can improve osteoblastic differentiation.

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Corresponding Author: Janeshin A., Dept. of Pediatric Dentistry, Guilan University of Medical Science, Rasht, Iran. Email: atousa_343@yahoo.com Tel: +98-13-33486406

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Introduction

Tooth formation is an advancing process that is regulated by reciprocal interactions between the epithelial and mesenchymal tissues. [1] These interactions stimulate a subpopulation of mesenchymal cells to dif-

ferentiate into odontoblasts, which, in turn, form the primary dentin. [2]

Cell division and the secretory activity of odontoblasts are limited in the adult pulp. But, after dental damage these processes may be reactivated, which

is subsequently associated with the formation of tertiary dentin including reactive and reparative dentin. In response to minor injuries, the primary odontoblast survive and reactionary tertiary dentin is formed; meanwhile, reparative dentinogenesis occurs after more intense injuries that lead to odontoblast death. [3] These all demonstrate that postnatal dental pulp contains cells with stem cell properties. [4] They are highly proliferative, clonogenic, and capable of regenerating a tissue; the properties that clearly define them as stem cells. Stem cells have several unique properties include the ability of self-renewal, proliferation, differentiation and they can give rise to specialized cell types. [4]

Stem cells are categorized as embryonic and adult. [5] In comparison to other types of adult stem cells used in tissue engineering, mesenchymal stem cells are more promising for therapeutic purposes. [6]

The most common source of mesenchymal stem cells is bone marrow; however, it has limitations such as the small number of cells obtained by biopsy and painful process. [7] Currently, several types of adult stem cells have been isolated from teeth, namely dental pulp stem cells (DPSCs), [4] stem cells from human exfoliated deciduous teeth (SHEDs), [8] periodontal ligament stem cells (PDLSCs), [9] stem cells from apical papilla (SCAPs), [10] and dental follicle progenitor stem cells (DFPCs). [11]

SHEDs are a population of highly proliferative cells which can differentiate into osteogenic and odontogenic cells, adipocytes, and neural cells. [12] They have several distinct advantages over dental pulp stem cells among which are increased cell population doublings, higher proliferation rate, ease of access, lack of tissue destruction at donor site, reduction or elimination of pain and discomfort, and the possibility of obtaining cells from young patients. [13] These cells can be regarded as the possible sources of stem cells for future tissue engineering applications. Tissue engineering is used in different fields such as bone regeneration. Another field of research following the isolation of stem cells from the desired source is to find the appropriate materials to add to the cell culture medium so that the most efficient material for the proliferation and differentiation of stem cells is introduced.

Vitamin D3 metabolites such as 1 α , 25-dihydrox-

yvitamin D3 are the key factors in regulation of bone metabolism. Studies have shown that 1 α , 25-dihydroxyvitamin D3 increases the ALP activity and osteogenic differentiation of normal permanent dental pulp as well as dental follicle stem cells. [14] Considering the differences between these cells and deciduous stem cells, the current study was designed to investigate the effect of 1 α ,25-dihydroxyvitamin D3 on osteogenic differentiation (alkaline phosphatase activity and alizarin red staining) of stem cells of exfoliated deciduous teeth.

Materials and Method

Fifteen sound exfoliated human deciduous teeth were collected from 10 healthy children aged 6 to 11 years old (4 girls and 6 boys). Prior to extraction, the patients received oral health education, professional prophylaxis was done for each patient, and they used 0.2% chlorhexidine mouthwash for one minute. Then, the teeth were extracted under sterile condition. The dental pulp was removed by using a Gracey curette and endodontic broach. It was immersed in a digestive solution containing 100 U/ml penicillin, 100 mg/ml streptomycin, and 500 mg/ml claritromycin in 4 ml 0.1 M phosphate-buffered saline (PBS) with the addition of 3 mg/ml type I collagenase and 4 mg/ml dispase, for 1 hour at 37°C. The solution was filtered through 70 μ m Falcon strainers (Falcon; Fisher, USA). After filtration, the cells were immersed in α -MEM (minimum essential medium) (Gibco; Germany), added with 10% fetal bovine serum (FBS) (Gibco; Germany), 100 μ m 2P-ascorbic acid, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μ g/ml streptomycin. The cell suspension was centrifuged and placed in flasks of 25 cm². The flasks were incubated at 37°C in a 5% CO₂, and the medium was changed twice a week. [15]

Osteogenic differentiation

After the third passage, the cells were isolated from the culture plate by using 25% trypsin-EDTA and were cultured for 24 hours. When the cell density reached 80%, they were used for osteogenic differentiation.

As the control group, the cells were cultured in osteogenic cell culture medium containing α -MEM (Gibco; Germany), 10% FBS, 50 μ g/ml L-ascorbic acid 2-phosphate, 50 mM β -glycerophosphate, and 0.1 μ M dexamethasone (Sigma). As the case group, the

cells were cultured in osteogenic culture medium supplemented with 100 nM1 α and 25(OH)2D3. The differentiation process was repeated 12 times. The medium was replaced with fresh medium every 3- 4 days. After 21 days of culture, alkaline phosphatase (ALP) activity was analyzed with a commercially available p-nitrophenylphosphate tablet set (Pars Azmoon; Iran). Likewise, the mineralization was analyzed by alizarin red staining (ECM 815; Millipore, USA). For alizarin red staining, the cell cultures were rinsed twice with FBS and fixed with 10% formaldehyde for 15 minutes at room temperature. Then, the cultures were stained with alizarin red solution for 20 minutes at room temperature. The excess dye was washed with distilled water. Digital images were taken of stained mineral deposits by using an inverted microscope. The total mineralized tissues were counted according to the manufacturer's instructions of alizarin red. Quantitative analysis of alizarin red staining was performed by determining the OD405 (optical density) values of a set of known alizarin red concentrations and comparing these values with the obtained values (Figure 1). [7, 16]

The results were analyzed by using t-test. For the analysis of normal distribution of data, non-parametric Kolmogorov-Smirnov test was used.

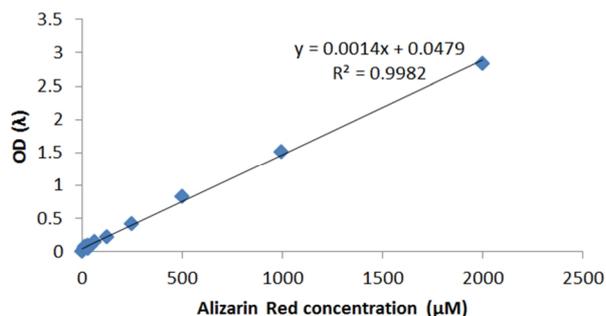


Figure 1: Concentration of alizarin red according to the standard optical density.

Results

Morphologic findings

The medium was observed by using an inverted light microscope on the days 1, 3, 7, and in the third passage. SHEDs were characterized with a typical fibroblast-like morphology. The morphology of the cells remained constant during cell passage (Figure 2).

Alkaline phosphatase activity

The effect of 1 α ,25(OH)2D3 on osteogenic differentia-

tion of SHEDs was analyzed by ALP activities. The results of Kolmogorov-Smirnov test indicated the normal distribution of data. Compared with the control group, significant increase was observed in ALP activity of SHEDs after being treated with 1 α ,25(OH)2D3 ($P= 0.002$) (Table 1).



Figure 2: Dental pulp stem cells of deciduous teeth at day 3 (100 \times magnification).

Table 1: The t-test results of alkaline phosphatase activity

Group	Number of examination	Mean (U/Lit) \pm SEM	P value
Control	12	44.85 \pm 2.87	0.002
Case	12	62.02 \pm 3.91	

Alizarin red staining

Quantification of alizarin red staining demonstrated that cells exposed to 1 α ,25(OH)2D3 induced higher mineralized nodules ($p < 0.001$) (Table 2, Figure 3).

Table 2: The t-test results of alizarin red staining

Group	Number of examination	Mean (μ M/MI) \pm SEM	P value
Control	12	531 \pm 10.78	<0.001
Case	12	727 \pm 35.53	

Discussion

This study aimed to isolate mesenchymal stem cells from exfoliated deciduous teeth in order to evaluate the effects of 1 α ,25(OH)2D3 on their osteogenic differentiation. Several studies have tried to find the appropriate method for regeneration of damaged bone which is an issue of paramount importance in dentistry. Regarding the systemic advantages of 1 α ,25 (OH) 2D3 in bone remodeling and wound healing, [17] in addition to its considerable role in tooth formation emphasized by *in vivo* and clinical studies, [14] the present study was designed to investigate the effect of 1 α ,25(OH)2D3 on osteogenic differentiation of SHED. SHEDs are postnatal stem cells with extensive prolife-

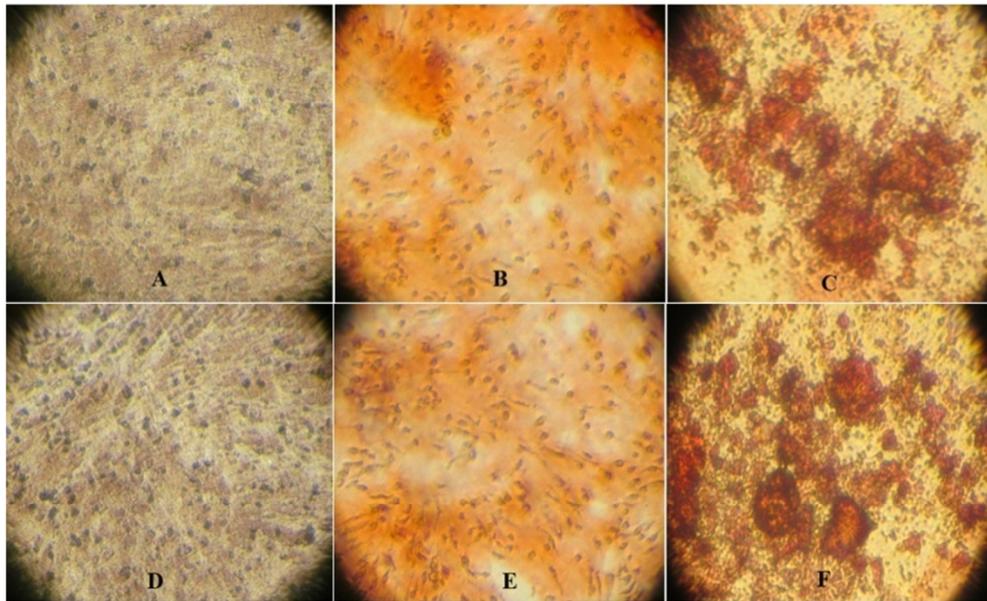


Figure 3: Alizarin red staining of mineralized deposits without (A, B, C) and with vitamin D metabolite (D, E, F). Osteogenic differentiation before staining (A and D) (40× magnification); after staining at 40X (B and E) and after staining at 400X magnification (C and F).

ration and differentiation potentials. Deciduous teeth, therefore, may be an ideal resource of stem cells to induce bone regeneration. [8] Mesenchymal stem cells have been extensively characterized *in vitro* by the expression of markers such as STRO-1, CD146 or CD44. [7] The International Society for Cellular Therapy declares that one of the required criteria to define human mesenchymal stem cells is expression of CD105, CD73, and CD90; in addition to lack of expression of CD45, CD34, CD14, CD11b, CD79a, CD19, and HLA-DR surface molecules. [18] Among the limitations of this study was the impossibility of performing flow cytometric analysis of cell surface due to the limited technical and financial resources. If these cells were end cells, they would not differentiate into other cell lines, so they were certainly considered stem cells.

Based on previous studies, differentiation of mesenchymal stem cells such as DPSCs and SHEDs into osteoblast-like cells is induced by dexamethasone, ascorbic acid, and β -glycerophosphate. [19] These osteogenic supplements are the most commonly used osteogenic inducer for human mesenchymal stem cells *in vitro*. $1\alpha,25(\text{OH})_2\text{D}_3$ was found to be more effective than dexamethasone in osteogenic differentiation of adipose-derived stem cells. [20] Osteogenic differentiation of bone marrow stem cells increased significantly in presence of $1\alpha,25(\text{OH})_2\text{D}_3$. [21] Osteoblastic

differentiation in DPSCs and DFPCs was stimulated by both $1\alpha,25(\text{OH})_2\text{D}_3$ and 25OHD_3 . [14] Therefore, despite the different categories of stem cells, the effect of $1\alpha,25(\text{OH})_2\text{D}_3$ was the same on osteogenic differentiation potential.

ALP activity is an early marker of osteogenic differentiation. The *in vitro* studies on osteoblastic cells revealed that $1,25(\text{OH})_2\text{D}_3$ increased the alkaline phosphatase activity. [22] It was reported that adding $1\alpha,25(\text{OH})_2\text{D}_3$ (10 and 100 nM) increased the ALP activity of dental pulp stem cells and dental follicle stem cells. [14] Likewise, the current study showed that $1\alpha,25(\text{OH})_2\text{D}_3$ (100 nM) enhanced the ALP activity in SHEDs. So, the response of stem cells in primary and permanent teeth are the same in this regard.

On the other side, Bakopoulou *et al.* detected that the ALP activity was significantly reduced in presence of HEMA (0.5mM and 0.1mM) and TEGDMA (0.25 mM, 0.1mM) in osteogenic culture differentiation, [16] which was unlike the effect of vitamin D3.

Khanna-Jain *et al.* reported that dental pulp stem cells and dental follicle stem cells formed mineralized matrix when treated with vitamin D3 metabolites in osteogenic differentiation culture. [14] Our results were consistent with Khanna-Jain *et al.*'s findings. [14] The alizarin red staining in our study demonstrated that the cells exposed to $1\alpha,25(\text{OH})_2\text{D}_3$ induced higher mineralized nodules.

Conclusion

The present study showed that osteoblast differentiation in SHEDs was stimulated by $1\alpha,25(\text{OH})_2\text{D}_3$. It can be concluded that $1\alpha,25(\text{OH})_2\text{D}_3$ improves the osteoblastic differentiation. Application of $1\alpha,25(\text{OH})_2\text{D}_3$ in tissue engineering is a suggestion which needs more investigations.

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Conflict of Interest

The authors of this manuscript certify no financial or other competing interest regarding this article.

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