

Effect of Calcitriol on Differentiation of Periodontal Ligament Stem Cells to Osteoblasts

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Abstract

Background: Periodontium may be able to respond to injuries by regeneration via the function of stem cells.

Objectives: This study sought to assess the differentiation of human periodontal ligament stem cells (PDLSCs) into osteoblasts in standard osteogenic medium and in a medium supplemented with 1,25-dihydroxyvitamin D3 (calcitriol).

Materials and Methods: In this experimental study, PDLSCs were isolated under sterile conditions by scraping the periodontal ligament tissues attached to the middle third of the root surface of extracted teeth, which were obtained from patients who were candidates for orthodontics therapy in the dental faculty at Hamadan University. The collected cells were cultured on four culture plates for 24 hours. Group 1 contained a basic medium (α -MEM, containing 10% fetal bovine serum (FBS), 5 mM β -glycerophosphate, and 50 μ g/mL l-ascorbic acid), supplemented with 10⁻⁸ M dexamethasone. Group 2 contained a basic medium supplemented with vitamin D3. Group 3 contained a basic medium supplemented with vitamin D3 and dexamethasone, Group 4 contained negative control cultures. Alizarin red staining (ARS), alkaline phosphatase (ALP) activity, and calcium content (CC) tests were performed to evaluate osteogenic differentiation of third passage cells in the developing adherent layer.

Results: Quantitative analysis of ARS demonstrated that mineralized nodule formation was highest in the group supplemented with calcitriol and dexamethasone ($P < 0.001$). Results of the ALP test on day 28 demonstrated the highest ALP activity in the group supplemented with calcitriol ($P < 0.001$). The amount of CC was lowest in the control group at all-time points, and was highest in the group supplemented with both calcitriol and dexamethasone on day 28 ($P < 0.001$).

Conclusions: The combination of calcitriol with dexamethasone, ascorbic acid, and beta-glycerophosphate (that is, the osteogenic medium) may be beneficial for differentiation of PDLSCs into osteoblasts.

Keywords: Calcitriol, Periodontal Ligament, Stem Cells, Cell Differentiation, Osteoblasts

1. Background

Periodontal treatment is performed with the aim of stopping progressive attachment loss. However, the ultimate goal of periodontal therapy is to regenerate the lost supporting structures. Successful periodontal regeneration includes re-establishment of the epithelial seal, formation of connective tissue fibers attached to the root surface, regeneration of acellular cementum on the root surface, and obtaining adequate alveolar bone height. To achieve this goal, several techniques have been recommended for use in the clinical setting, such as guided tissue regeneration (GTR); implantation of autografts, allografts or alloplastic materials; chemical root conditioning; use of growth factors; or a combination of these approaches (1, 2).

one of the newest methods for regenerating lost tissue is using stem cells. Mesenchymal stem cells have the poten-

tial to differentiate to different cell types (chondrocytes, osteoblasts, fibroblasts, adipocytes) and many mesenchymal tissues. These cells are present in many tissues and organ (3).

Periodontal ligament (PDL) contains pluripotent stem cells as a source of periodontal tissue regeneration. It is believed that periodontal regeneration can be successfully attained via the migration of periodontal ligament stem cells (4, 2). Seo et al. verified that only some of the progenitor cells of PDL can be considered stem cells. These periodontal adult stem cells have the phenotypic, morphological, and proliferative characteristics of adult mesenchymal stem cells (5). These cells are capable of promoting tissue turnover and homeostasis, serving as a source of renewable progenitor cells that generate osteoblasts, cementoblasts, and fibroblasts throughout adult life. In response to inflammatory processes, these stem cells are ac-

tivated by mediators when periodontal tissues are damaged (6, 7). Based on this capability, new methods of tissue regeneration have been developed and some products have been introduced, such as bone morphogenetic proteins and enamel associated proteins, in attempts to regenerate periodontal tissues (8). However, the exact cells responsible for the regeneration of the periodontium have yet to be determined. It has been shown that many of the events required for the development of periodontal tissues are similar to those required for regeneration of periodontal tissues. In both situations, appropriate cells must be attracted to and attach to the target site. To provide an environment appropriate for cell proliferation and differentiation, an appropriate matrix must be secreted by cells for them to have the capacity to function as PDL fibroblasts, osteoblasts, or cementoblasts (9).

Periodontal ligament stem cells are a class of dental ectomesenchymal stem cell that is isolated from the root surface of extracted teeth. These cells can be isolated as plastic-adherent, colony-forming cells, but under in vitro conditions, they display a low potential for osteogenic differentiation. PDL stem cells differentiate into cells or tissues very similar to the periodontium (10). PDL stem cells have demonstrated a capacity for tissue reconstruction and periodontal repair when transplanted into immunocompromised rats and mice (5). Recently, PDL stem cells also have been isolated from sheep and pigs (11, 12). It has been shown that a functional periodontium can be established successfully by using PDL stem cells (13).

Calcitriol, also known as 1, 25-dihydroxycholecalciferol or 1, 25-dihydroxyvitamin D₃, is the hormonally active metabolite of vitamin D known for its important role as a regulator of mineral and bone metabolism. It has a high affinity for the nuclear vitamin D receptor (14, 15). It can inhibit the proliferation and promote the differentiation of many cell types, especially tumoral cells (16, 17). Epidemiologic and experimental studies have also demonstrated the anti-tumor effects of calcitriol (18) because it inhibits cell proliferation (17, 19, 20), arrests cell cycles (16, 21), promotes apoptosis (22, 23), and induces cell differentiation (24). Vitamin D receptors are present in most if not all cells in the body. Additionally, experiments using cultured cells have demonstrated that vitamin D has significant effects on differentiation of many types of cells into osteoblasts. These findings suggest that vitamin D may play a role in mineral homeostasis and bone metabolism (25, 26).

2. Objectives

This is an active area of research. Thus, the present study was conducted to assess the in vitro effect of calcitriol on differentiation of PDLSCs into osteoblasts.

3. Materials and Methods

3.1. Isolation of Periodontal Stem Cells

Stem cells were isolated from the roots of 15 clinically intact premolars from young patients who had undergone orthodontic treatment. Patients were visited and scaling was done 5 to 7 days before teeth extraction (5). If visible plaque existed before extraction, polishing of the tooth to be extracted was done again. In order to minimize the risk of contamination of specimens, prep and drape were done after local anesthesia. Also, patients were asked to rinse diluted povidone iodine with normal saline (1:1), because this mouth rinse is faster and has stronger effect. Instruments were precisely sterilized, and surgeon and assistants wore masks, surgical gowns, and sterile gloves. Extraction had to be done with minimal wobbling. The surgeon held the coronal portion of the tooth with forceps and separated the root part using a disc and high-speed hand piece under copious saline irrigation, to avoid over-heating and subsequent cell damage. After cutting through the tooth thickness, the surgeon used pliers to break the root part. One assistant opened a sterile tube containing alpha-modified eagle's medium (α -MEM), and the root was immediately placed in the tube. Care was taken not to contact any surfaces, even the outer surface of the tube. The tube was covered and sealed immediately with a wax sheet (27). The method used to isolate the stem cells was in accord with that described by Gay et al. as follows:

By using a surgical scalpel, the periodontal ligament tissues only attached to the middle third of the root surface were removed to avoid contamination by gingival and pulpal cells. Scraped periodontal ligament cells were transferred to a plate containing α -MEM and 15% fetal bovine serum (FBS), and enzymatically digested for 1 hour at 37°C using 3 mg/mL collagenase type I solution. Then the samples were centrifuged at 400 g for 10 minutes and plates were expanded with α -MEM containing 15% FBS and 1% antibiotics in 6-well plates and cultured at 37°C and 5% CO₂. Adherent cells with 80 - 90% confluence were washed twice with phosphate-buffered saline (PBS) and detached from the culture surface using 0.25% trypsin-EDTA solution (Gibco, Germany) and plated in tissue culture polystyrene flasks at 5×10^3 cells/cm² on day 7. Primary cultures of PDLSCs mainly consisted of colonies of bipolar fibroblastoid cells, reaching a confluent growth-arrested condition, after sub-cultivation and proliferating within a population doubling time of 48 hours (28).

3.2. Osteogenic Differentiation

Cells in the developing adherent layer were used for osteogenic differentiation after detachment with 0.25% trypsin-EDTA solution at the third passage. The collected

cells were cultured in four culture plates for 24 hours. The culture medium was changed when the confluence of cells reached 80%, as follows:

Group 1: contained basic medium (α -MEM containing 10 % FBS, 5 mM β -glycerophosphate, and 50 μ g/mL l-ascorbic acid) supplemented with 10⁻⁸ M dexamethasone.

Group 2: contained basic medium supplemented with vitamin D3.

Group 3: contained basic medium supplemented with vitamin D3 and dexamethasone.

Group 4: negative control cultures, which were maintained in α -MEM containing 10% FBS without osteogenic supplements. (Vitamin D3 at 10 ng/mL was dissolved in α -MEM).

Cells were re-fed every 3 days, and then ALP activity was tested on days 7, 14, 21, and 28. The calcium content (CC) test was done on days 7, 14, 21, and 28, and the alizarin red staining (ARS) was performed on day 28 of the subculture growth. Each group was tested three times and the mean value was reported.

3.3. Alizarin Red Staining (ARS)

Mineralization of the cell layer was examined using ARS. The cells were rinsed twice in PBS, fixed in 10% formaldehyde, and incubated at room temperature for 15 minutes. After rinsing with distilled water, the plates were stained with 1 mL/well ARS solution (Millipore Co.), incubated at room temperature for at least 20 minutes, and rinsed with deionized water, after which 1 - 1.5 mL of water were added to each well. Differentiated cells containing mineral deposits were stained bright red by the ARS.

3.4. Quantitative Analysis of ARS

A total of 400 μ L of acetic acid were added to each well and incubated for 30 minutes while shaking. With the aid of a cell scraper, the cells were gently scraped from the plate, and the cells and acetic acid were transferred to a 1.5 mL micro centrifuge tube. They were then heated to 85°C for 10 minutes, after which the tube was transferred to an ice pot for 5 minutes. The slurry was centrifuged at 18000 g for 15 minutes to make a standard solution. The 10x ARS dilution buffer was diluted 1:10 in distilled H₂O, and the 40 mM of ARS solution were diluted 1:20 in 1x ARS dilution buffer. This gave a 2 mM working stock. Standards could be prepared in a 'high range' or 'low range' set. Preparation of the 'high range' set was done by diluting the 2 mM working stock in 2-fold serial dilutions in 1.5 mL micro centrifuge tubes. To prepare a 'low range' set, we began by first diluting the 2 mM working stock 1:66 (15 μ L 2 mM ARS + 985 μ L 1x ARS dilution buffer) to achieve a 30 μ M working stock. Preparing the 'low range' set was done

by further diluting this 30 μ M working stock in 2-fold serial dilutions in 1.5 mL micro centrifuge tubes. The blank consisted of just 1x ARS dilution buffer from which 400 μ L of the supernatant was removed and transferred to a new 1.5 mL micro centrifuge tube. After centrifugation, the pH was neutralized and 150 μ L of the standard sample were added to an opaque-walled, transparent-bottom, 96-well plate. It was read at OD405. Alizarin red concentration vs. OD405 was plotted. The alizarin red quantitative data set is shown in Figure 1. Each group was tested three times and the mean value for each group was reported.

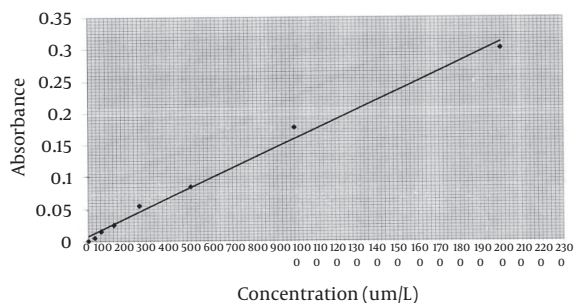


Figure 1. Alizarin Red Quantitative Data Set

3.5. Alkaline Phosphatase (ALP) Activity

In addition to ARS, assessment of osteoblastic differentiation was done by measuring ALP activity in the culture medium using an ALP kit (Pars Azmun Co, Iran, Tehran). PDLSCs were cultured as described above, and on days 7 and 14 the used medium in each group was collected and stored at [U+2012] 20°C. To analyze ALP activity, the collected medium was diluted in a solution containing four parts of buffer solution (1 M diethanolamine and 0.5 mM MCl₂) and one part of substrate solution (10 mM p-nitrophenyl phosphatase). ALP activity was determined colorimetrically with p-nitrophenyl phosphatase as a substrate.

3.6. Calcium Content (CC) Test

On days 7, 14, 21, and 28, the cells were washed in PBS. The 0.5 mL PBS solution containing 5% Triton X-100 was added to each well. With the aid of a cell scraper, the cells were scraped from the plate and stored at [U+2012] 20°C. Then, the cells were frozen and re-frozen 3 times, and finally the CC in the samples was measured by the o-cresolphthalein complexone method on days 7, 14, 21, and 28 of subculture growth. The test was done according to the instructions of Pars Azmun Co.

3.7. Statistical Analysis

Statistical analysis was done using SPSS version 17 software. Deliberation was done using 6×3 factorial (six group's \times three periods). The data then were subjected to one-way analysis of variance (ANOVA), and the differences between the groups were compared using Tukey's post hoc test. A normal distribution of data was tested by the non-parametric method using the Kolmogorov-Smirnov test.

4. Results

4.1. Results of Quantitative Analysis of ARS

Quantitative analysis of ARS on day 28 demonstrated that mineralized nodule formation was the highest in the group supplemented with vitamin D3 and dexamethasone (Figure 2). The difference between the vitamin D3 group and the control group was not significant ($P = 0.625$), but the ANOVA test showed that the difference between the vitamin D3 + dexamethasone group and all other groups was significant ($P < 0.001$).

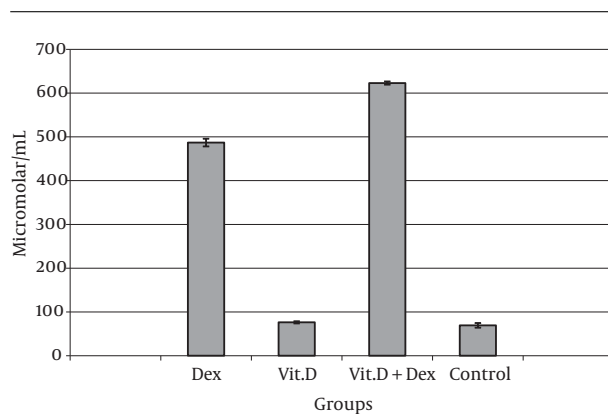


Figure 2. Quantitative Analysis of Alizarin Red Staining in Different Groups

4.2. Results of ALP Activity Test

The ALP activity was measured after 7, 14, 21, and 28 days of subculture growth to quantify osteoblastic differentiation (Figure 3). After 28 days of subculture growth, the highest ALP activity was seen in the group supplemented with vitamin D3. The differences between the vitamin D3 and vitamin D3 + dexamethasone groups was not significant, but the ANOVA test showed that there was a significant difference in this regard between all other groups ($P < 0.001$).

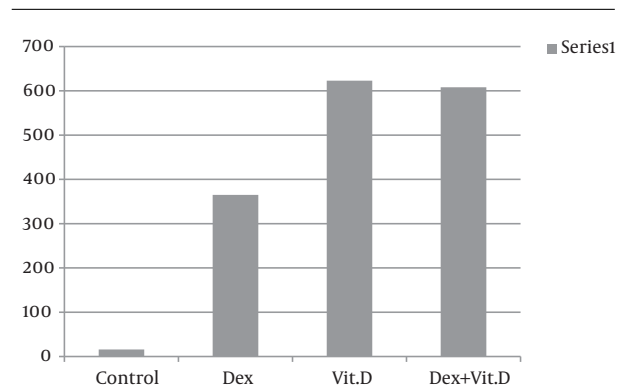


Figure 3. ALP Activity Test in Different Groups After 28 Days of Subculture Growth

4.3. Results of CC Test

The CC test was done at 7, 14, 21, and 28 days of subculture growth and demonstrated that the amount of calcium in the group supplemented with dexamethasone + vitamin D3 was the highest (Figure 4). The ANOVA test showed that there was a significant difference between the control group and all other groups ($P < 0.001$). Moreover, the difference between the vitamin D and vitamin D + dexamethasone groups was significant as well ($P < 0.001$), but the difference between the dexamethasone and vitamin D + dexamethasone groups was not significant ($P = 0.5$).

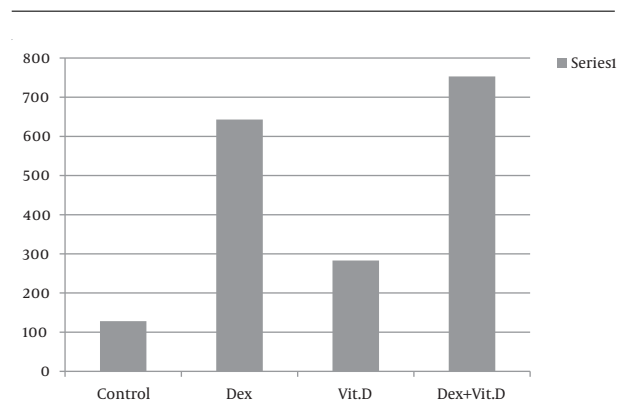


Figure 4. Intracellular Calcium Deposition in Different Groups After 28 Days of Subculture Growth

4.4. Changes in ALP Activity

There was a slight increase in ALP activity in the control group during the study (Figure 5). In this group, the level of ALP on days 7, 14, 21, and 28 was 12.5, 14.0, 14.5, and 16.0 U/L, respectively. The amount of ALP in the dexamethasone group on days 7, 14, 21, and 28 was 127, 613, 583, and 365 U/L, respectively. These values in the vitamin D3 group were 133,

620, 621, and 623 U/L, respectively. In the vitamin D3 and dexamethasone group, these values were 173, 284, 487, and 608 U/L, respectively.

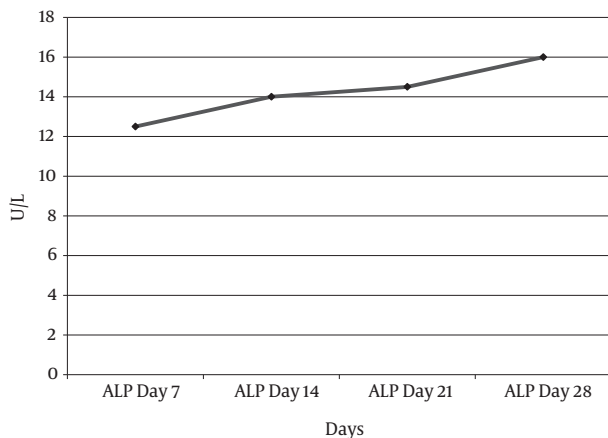


Figure 5. Changes in ALP Activity in the Control Group

5. Discussion

Bone regeneration is an important topic, and many researchers have focused on methods and materials to achieve this goal. Considering the optimal properties of systemic vitamin D3 and its role in bone remodeling, decreasing the risk of bone fracture, and enhancing wound healing, this study sought to assess the effect of vitamin D3 on differentiation of PDLSCs into osteoblasts. In order to study the effects of calcitriol on osteogenesis, we used ALP activity, and CC and ARS tests at 7, 14, 21, and 28 days. ALP is present in the cell membrane of osteoblasts, and any increase in its activity indicates the differentiation of stem cells into osteoblasts (9). Although the combination of vitamin D3 and dexamethasone increased the activity of ALP in our study, the addition of vitamin D3 alone showed the same effect as well. At 7 and 14 days, dexamethasone increased the activity of ALP, but it later decreased at 21 and 28 days; whereas ALP activity did not decline in the vitamin D3 group after day 14. Our results regarding the effect of calcitriol on increasing ALP activity were somewhat in accord with those of Geng et al. They evaluated the effect of 25-hydroxyvitamin D3 on proliferation and osteoblastic differentiation of human marrow stromal cells, and demonstrated inhibition of proliferation and promotion of differentiation by calcitriol (29). Although type of stem cells used in their study was different from ours, the same effects were noted for calcitriol on these cells as in our study.

Moreover, Tang and Meng demonstrated that osteogenic induction significantly enhanced the proliferation of human periodontal ligament cells, whereas calcitriol inhibited their proliferation (25). These findings are somewhat in accord with our results as well. Zur Nieden et al. in 2003 showed differentiation of embryonic stem cells into mineralized osteoblasts due to exposure to ascorbic acid, beta-glycerophosphate, and 1-alpha, 25-OH vitamin D3. ALP activity increased at of culture growth, and mineralized cells were detected by histochemical staining. Expression of osteopontin and osteonectin (involved in the process of binding calcium ions and hydroxyapatite during the mineralization process) was also enhanced at the beginning of the second culture week. ALP activity increased again at the beginning of the third week of culture growth and was enhanced again at later stages of culture growth at days 27 - 34. Although their study was conducted on mouse embryonic stem cells, results similar to our outcome were obtained regarding the effect of 1-alpha, 25-OH vitamin D3 on induction of osteoblast differentiation (30).

Most of the actions of vitamin D3 are mediated through a nuclear factor known as the vitamin D receptor (VDR) (31). Upon entering the nucleus of a cell, 1, 25-dihydroxyvitamin D3 adheres to the VDR and promotes its association with the retinoic acid X receptor (RXR). In the presence of 1, 25-dihydroxyvitamin D3, the VDR/RXR complex binds small sequences of DNA, known as vitamin D response elements (VDREs), and initiates a cascade of molecular interactions that modulate the transcription of specific genes. More than 50 human genes are known to be regulated by 1, 25-dihydroxyvitamin D3 (14). Tang and Meng demonstrated that calcitriol significantly increased the level of VDR by three-fold (25).

The special kit used for ARS in this study enabled examining the quantitative ARS to compare the amount of mineralized nodules formed. The results of ARS showed that dexamethasone had a significant effect on formation of mineralized nodules, indicative of osteogenic differentiation, which is in accord with the results of Langenbach and Handschel (32); vitamin D3 alone had no significant effect in this regard. The combination of vitamin D3 and dexamethasone again significantly enhanced osteoblastic differentiation. These results were confirmed by the CC test as well. A significant increase was observed in the CC for all groups, in contrast to the control group. However, the combination of vitamin D3 and dexamethasone caused a greater increase in this regard. Compared to vitamin D3 alone, dexamethasone was more effective on the CC of the cells, and based on our results presence of vitamin D3 was necessary for this effect.

Future studies are recommended to assess the effect of different doses of tested materials, in order to find the

most effective dosage. Moreover, animal studies are required to better elucidate the effect of calcitriol on PDLSCs, and to obtain results that can be more generalized to the clinical setting.

5.1. Conclusions

The combination of vitamin D3, dexamethasone, ascorbic acid, and beta-glycerophosphate (the osteogenic medium) may enhance the differentiation of PDL stem cells into osteoblasts.

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Footnote

Authors' Contribution: Sara Soheilifar: guide prof; designing the study, tips on data collection and writing the paper; Iraj Amiri: advisor prof, all stages of cells isolation and culture; Mohsen Bidgoli: assisting in writing the paper; Janet Moradi: assisting in writing the paper; Morad Hedayatpanah: corresponding author; Mohammad Ali Momeni: data collection.

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