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The Potential of Human-derived Periodontal Ligament Stem Cells to Osteogenic Differentiation: An *In vitro* Investigation

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Abstract

Background: Periodontal ligament stem cells (PDLSCs) are considered as a type of mesenchymal stem cell that is beneficial target for numerous clinical applications in periodontal tissue regeneration therapy.

Materials and Methods: This study examined the effects of dexamethasone (Dex) on human PDLSCs *in vitro*. PDLSCs obtained from the roots of patient's teeth were cultured with Dex (0.01 µM), and their proliferation was measured. The osteogenic differentiation was assessed by alkaline phosphatase (ALP) activity and Alizarin Red-S staining for calcium deposition.

Results: After the administration of 0.01 µM Dex, the activity of ALP increased significantly. Furthermore, mineralized nodule formation showing the intracellular calcium deposition was significantly higher in the Dex-treated cells than that of the control cells.

Conclusion: Collectively, Dex has positive effects on osteogenic differentiation of human PDLSCs in vitro. It is suggested that PDLSCs may serve as a potential material for periodontal tissue regeneration.

Keywords: Periodontal ligament stem cells; Osteogenic differentiation; Dexamethasone

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Introduction

Periodontal disease or periodontitis is a chronic infection associated with different organs consisting of epithelial tissue and soft and mineralized connective tissues, resulting in inconvenience effects of lost tooth and emerging systemic diseases (1,2). This is an inflammatory disease as the result of interaction between subgingival plaque microbiota and host responses (3). It is estimated that this high prevalent disease may affect 90% of the worldwide population. Periodontal diseases are associated with adverse pregnancy outcomes, coronary heart disease (4), stroke, pulmonary disease, and diabetes (5). Therefore, it seems critical to manage treatment planning. To date, several therapeutic ways, such as implantation of autografts, allografts and alloplastic materials, chemical root conditioning, growth factors, guided tissue regeneration (GTR), and combination of these approaches have been used in clinical practice (6). All therapies aim to reduce further attachment loss, connective tissue damage and therefore improving periodontal regeneration and healing (7). Current therapies is based on the Melcher's hypothesis, "compartmentalization". He proposed that the connective tissue between tooth and periodontal tissue are categorized into four groups: the lamina propria of the gingiva (gingival corium), the periodontal ligament (PDL), the cementum, and the alveolar bone. These four types of cells have the potential to recreate from their original and undifferentiated cells and repopulate in the wound

area (8). Therefore, only PDL are the cells that could be repopulated for periodontal regeneration.

PDL is a connective tissue that consists of unmineralized network of collagen fibers (9), and locates between tooth root and alveolar bone (10). It covers the root of the tooth, functionally maintaining teeth in alveolar bone (11) and contributes in periodontal regenerating in response to wounding, periodontitis, and orthodontic tooth movements after treatment. The presence of pluripotent stem cells within PDL make it as a suggestive tissue involving in preserving tissue homeostasis and periodontal regeneration. Periodontal regeneration can occur following the migration of periodontal ligament stem cells (PDLSCs) and their differentiation into cementoblasts, osteoblasts and fibroblasts, and finally develop into extracellular matrix similar to periodontal tissue (12). This ability has been attributed to the pluripotency of PDL-derived stem cells originating from the neural crest and shed new lights on therapeutic approaches (13).

In the current study, stem cells obtained from patient's premolars-derived PDL were investigated for *in vitro* evaluation of the ability of PDL to differentiate into osteoblasts and calcified deposits formation. For this purpose, morphological features and calcified nodule arrangement were monitored under selective cultivation conditions.

Materials and Methods

Patient selection

PDLSCs were obtained from the roots of 17 clinically healthy first premolars from young individuals undergoing tooth extraction due to orthodontic reasons. Routine dental cleaning including scaling and polishing was performed before teeth extraction. Patients were advised to brush their teeth thoroughly; dental plaques were removed by teeth repolishing immediately before teeth extraction.

Isolation, culture, and preparation of stem cells

Preparation and drape, and local anesthesia were performed. There are several procedures to minimize the risk of transmitted infection: rinsing patient's teeth by diluted povidine-iodine, sterilizing instruments precisely, washing sterile gloves with normal saline and wearing mask and surgical gown. After extraction, the coronal portion of root was held by a sterile pliers and the crown was separated by a disk. Copious amount of saline was used for rinsing in order to reduce temperature and avoid cell damage. Cells should not be damaged by inadvertent movements of pliers tip. The root was immediately entered into tube containing Hanks' Balanced Salt Solution (HBSS) medium and tube was covered and sealed by wax sheet. Only periodontal ligament tissues attached to the middle third of the root surface were scraped with surgical scalpel.

The stem cells culture was conducted according to Gay *et al*. as follows (14). PDL cells were scraped from premolars and enzymatically digested for 1h at 37 °C in a solution of 3 mg⁄ml collagenase type I (Sigma Co., Germany). Then the samples were centrifuged at 400 ×g for 10 min. PDL cells were grown in suspension culture at 37 °C in 5% humidified CO2 in α-MEM (Minimum Essential Medium Eagle- Alpha Modification) (Gibco Co., Germany) containing 15 % FBS (Gibco Co., Germany), 100 U/ml penicillin- G, 100 µg/ml streptomycin, and 0.25 µg/ml fungizone (Gibco Limited, Uxbridge, United Kingdom) in a 6 well plate as primary culture. When the cells reached 80-90% confluence, cultures were washed twice with phosphate buffer saline (PBS) and the cells were trypsinized with 0.25% trypsin in 1mM EDTA (Gibco, Germany). At day 7, cells reached 80-90% confluence, adherent cells were centrifuged and resuspended in serum supplemented medium in tissue culture polystyrene flasks at a density of 5×10^3 cells/cm^2.

Flow cytometry

The second passage of the cells were used for flow cytometry analysis following incubation with primary FITC or PE conjugated monoclonal antibodies (CD90, CD105, CD146, CD31, CD34, CD45 Ab) (R&D Systems Co., China) and then adding fluorescein-conjugated secondary antibody. The cells were washed twice and fixed with 4% paraformaldehyde. The cell surface markers (CD90, CD105, CD146 positive and CD31, CD34, CD45 negative cells) were analyzed and sorted using a FACSCalibur flow cytometer (Becton Dickinson) by collecting 10,000 events.

Figure 1. Migration of numerous fibroblastoid cells from the explants after 3 days of culture.

Osteogenic differentiation

Adherent cells obtained from second passage wereused for osteogenic differentiation. The cells wereplated in tissue culture dishes in osteogenic medium,including modified minimum essential medium (α-MEM) supplemented with 10 % FBS, 10^{-8} M (0.01μM) Dex (Iran Hormone Pharmaceutical Co, Iran), 5 mM β-glycerophosphate and 50 μg/ml l-ascorbic acid.

Cultures in α-MEM containing 10% FBS without osteogenic supplements were considered as control. The cells were then refed every 3 days until calcified nodule-like structures were observed (usually after 4 weeks). These nodular aggregates of cells were morphologically evaluated by inverted microscope and subsequently prepared for histochemical analysis.

Histochemical Analysis

The presence of calcified nodules and mineralization were confirmed using Alizarin Red-S staining. After rinsing the cells twice with PBS, ice-cold 70% ethanol was added to fix the cells. Then the cells were washed with deionized water and stained with 40 mM Alizarin Red-S (pH 4.2) (Sigma-Aldrich, Germany) for at least 10 min at 37 °C. Non-specific stain was removed by washing with PBS. The plates were then air dried and visualized under inverted microscope. The calcium content was measured using o-cresolphthalein complex method at 7, 14, 21, and 28 days of subculture according to the manufacturer's instruction (Pars Azmun, Iran).

Moreover, osteoblastic differentiation was assessed by measuring alkaline phosphatase (ALP) activity in the culture medium using alkaline phosphatase assay kit (Pars Azmun, Iran). Each assay was repeated three times and the mean value of the results was recorded.

Statistical analysis

Statistical analysis was performed using SPSS version 17. The statistical method was selected according to verification of normal distribution of varibles using Kolmogorov-Smirnov test. Difference in continuous variables in two groups was measured by Student t test. One-way analysis of variance (ANOVA) and the Tukey's post hoc were used to compare the differences between more than 2 groups. P-value less than 0.05 was considered as statistical significance.

Results

In this study, a pluripotent stem cell population derived from human PDL. At the bottom of the plates the visible cells were mesenchymal type stem cell, adherent spindle shaped and fibroblast-like cells. Primary cultures of PDLSCs mainly consisted of colonies of bipolar fibroblastoid cells. After subcultivation, the cells were proliferated with a population-doubling time of 48 h and reached a confluent growth-arrested state. After three days of culture, numerous fibroblastoid cells migrated from the explants (Figure 1). Flow cytometry analysis revealed that the cells were positive for CD90, CD105 and CD146 and negative for CD31, CD34, and CD45 as other PDLSCs. After seven days 80-90% confluent cells were passaged and passage 2 was seeded for differentiation (Figure 2).

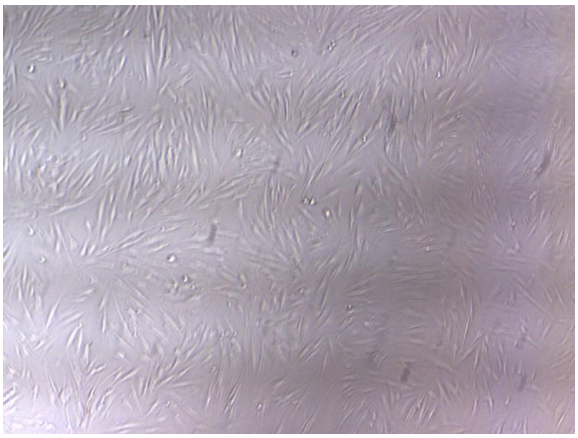

Figure 2. Developing and growing adherent cell layer used for osteogenic differentiation

Morphological analysis of PDLSCs showed oateoblast differentiation (Figure 3).

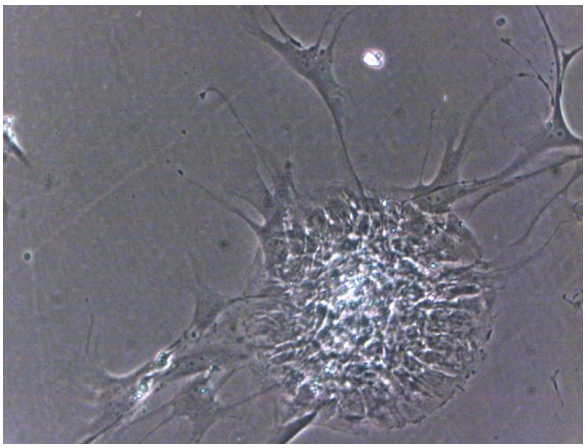

Figure 3. The presence of living cells organized in calcified nodule-like structures.

The results showed the presence of living cells organized in calcified nodule-like structures. The nodular aggregates of cells in differentiating cultures stained with Alizarin Red-S demonstrated that the deposits observed are calcified. Extensive osteogenic differentiation was observed only in PDL-MSCs exposed to osteogenic conditions.

This is indicated by calcium deposition (Figure 4). While there is no mineralization in the cells cultured in control medium (Figure 5). On the other hand, the highest mineralization was observed on 28th day in Dex-treated PDLSCs compared to what has been found in control group (480± 62 vs. 76± 16 µM/ml, $p<0.0001$) (Figure 5). The lowest amount of intracellular calcium deposition was observed in control group. An increasing trend of calcium deposition was observed only in Dex treated PDLSCs (Figure 6). On the 7th day, ALP activity of PDLSCs was different in Dex group compared to that of the control group but this difference was not statistically significant. Interestingly, ALP activity increased significantly on 14th day in Dex group compared to that of the control group (620±66 vs. 28±14 U/L, $P<0.0001$) (Figure 7).

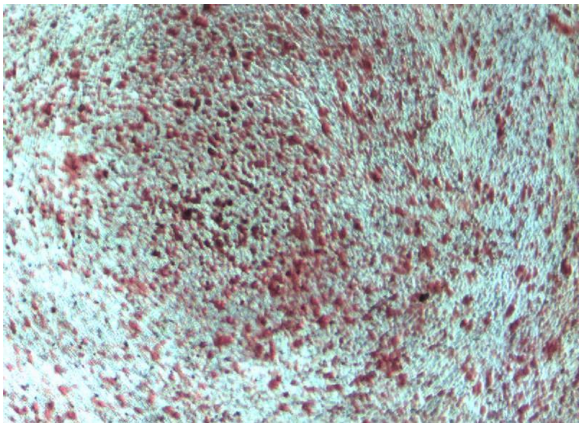

Figure 4. The deposits observed in calcified nodule-like structures stained with Alizarin Red-S which indicates calcium deposition

Discussion

The aim of the present study was to investigate the capability of human periodontal ligament stem cells for osteogenic differentiation. Numerous biological and clinical studies provide the evidence that PDL are capable of differentiating into calcified-forming osteoblasts under certain conditions. The first human dental stem cells were obtained from dental pulp tissue of extracted third-molar teeth that are able to form a dentin/pulp-like complex. They share similar features with bone marrow mesenchymal stem cells (BMSCs). The dental pulp stem cells (DPSCs) produced dense calcified nodules and did not form adipocytes, whereas BMSCs were calcified throughout adherent cell layer with adipocytes (15). The first isolation of periodontal ligament stem cells occurred in 2004 by Seo *et al*. They isolated PDLSCs from surgically extracted human third molars. They found that PDLSCs can express mesenchymal stem cells marker such as STRO-1 and CD146/MUC18. Moreover, PDLSCs are able to differentiate into cementoblast-like cells, adipocytes, and collagen-forming cells under certain culture conditions. The *in vivo* analysis on immunocompromised rodents confirmed the PDLSCs potential to produce a cementum/PDL-like structure and involved in periodontal tissue repairing. Therefore, PDL could be considered as a therapeutic strategy for periodontal diseases-derived destroyed tissues (16).

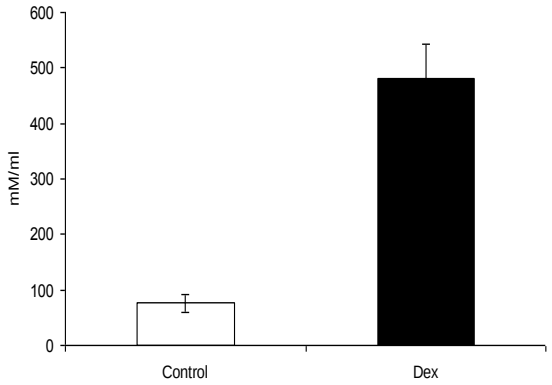

Figure 5. Quantitative analysis of Alizarin Red staining of PDLSCs

The ability of osteogenic differentiation from PDLSCs was previously discussed (17). In our analysis, osteogenic differentiation was induced by culture medium containing ascorbic acid which induces differentiation.

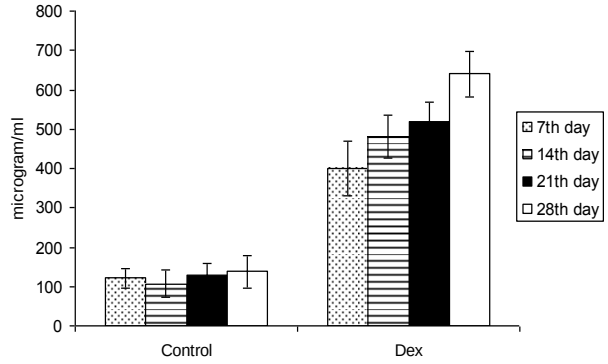

Figure 6. Kinetic pattern of intracellular calcium deposition of PDLSCs under two condition media in 7, 14, 21, and 28 weeks.

Several studies demonstrated the osteogenic differentiation from periodontal tissue by different factors and mediators: IL-6 may act as an osteolytic factor and mediate osteoblastic differentiation from periodontal ligament cells (18), this form of differentiation could be induced by simvastatin which inhibits mevalonate pathway and result in human PDL cells proliferation and osteogenic differentiation (19). The impact of icariin on promotion of human

periodontal ligament cells (hPDLCs) differentiation into osteoblast was confirmed by high osteogenesis gene expression in a study performed by Ding *et al* (20). The osteogenic differentiation was observed in our study under culture condition treated with Dex. There is some evidence that used Dex for inducing osteogenesis in stem cells. Dex is an osteogenic inducer that are involved in osteoblastic differentiation in human clonal bone marrow cell line (21).

Dex can stimulate the *Runx2* (Runt-Related Transcription Factor 2) via β-catenin-dependent pathway, TAZ (transcriptional co-activator with PDZ-binding motif), and MKP-1 (mitogen-activated protein kinase (MAPK) phosphatase) activation (22). *Runx2* participates in bone formation, regulation of osteoblast differentiation, and leads to increased osteogenic gene expression (23). It was found that Dex is an important factor for mineralization of human mesenchymal stem cells (hMSCs), therefore, hMSCs can act as a candidate source of osteogenesis under optimum culture condition (24). The osteogenic differentiation ability of PDLSCs and pulp of human exfoliated deciduous teeth (SHED) was analyzed by Chadipiralla *et al* (25). They demonstrated the osteogenic ability of PDLSCs and SHED in medium supplemented with Dex and their potential for *in vivo* bone regeneration. Thus PDLSCs have a favor capability in clinical and therapeutic applications.

The main necessity of periodontitis treatment is mineralized periodontal tissue reconstruction and its regeneration (26), therefore, osteogenic differentiation seems to be a critical achievement. The importance of PDLSCs regeneration and osteogenic differentiation were demonstrated in many studies.

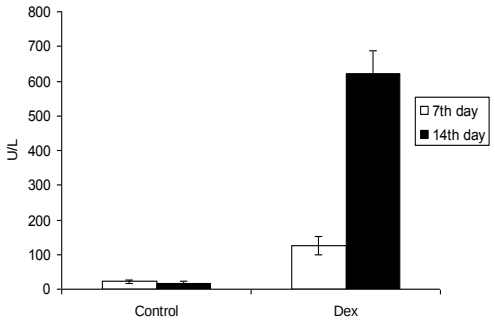

Figure 7. Alkaline phosphatase activity in supernatant of PDLSCs cultures on 7th and 14th days

Encapsulated form of PDLSCs was applied for tendon regeneration after 4 weeks differentiation. High expression of tendon regeneration-relating markers (Scx, DCn, Tnmd, and Bgy) confirm their positive effect. The consistent results derived from *in vivo* analysis made them as a therapeutic source for tendon engineering (27). IL-10, an anti-inflammatory cytokine, involves in bone mass maintaining and regulating osteoblast production. Thus, IL-10 could be of great benefit in bone loss diseases therapy (28). There are some compounds such as NELL1 that can induce osteogenic differentiation of hPDLSCs, thus, they are associated with periodontal regeneration gene therapy (29).

In summary, the above findings suggest that human PDLSCs have osteogenic differentiation ability and has direct effect on periodontal-relating diseases. Alizarin Red-S staining proved that mineralization occurred in the cell layers during osteoblast differentiation from PDLSCs in cultures containing osteogenic medium. Our study comes to same conclusion as others in which human periodontal ligament tissue-derived MSCs are able to differentiate into the calcified structure and/or osteoblastic lineage. Therefore, PDLSCs could be considered as a gold candidate for periodontal regeneration and its associated diseases.

Conflict of Interest

The authors declare that they have no conflict of interest in this work.

Authors' Contributions

HB and MA designed the study, MT analyzed data and reviewed the manuscript. RA and OA performed the experiment and wrote the first draft of the manuscript. MA and HB contributed to experimental design, interpreted data and reviewed the manuscript. All authors reviewed and approved the final version of the manuscript

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