Original Article

Comparison of Periodontal Ligament Stem Cells Isolated from the Periodontium of Healthy Teeth and Periodontitis-Affected Teeth

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Abstract

Objectives: Stem cell (SC) therapy is a promising technique for tissue regeneration. This study aimed to compare the viability and proliferation ability of periodontal ligament stem cells (PDLSCs) isolated from the periodontium of healthy and periodontitis-affected teeth to obtain an autologous, easily accessible source of SCs for tissue regeneration in periodontitis patients.

Materials and Methods: The PDLSCs were isolated from the roots of clinically healthy premolars extracted for orthodontic purposes and periodontally involved teeth with hopeless prognosis (with and without phase I periodontal treatment). Cells were cultured and viability and proliferation ability of third passage cells in each group were evaluated using the methyl thiazol tetrazolium assay. The results were statistically analyzed using t-test.

Results: No SCs could be obtained from periodontitis-affected teeth without phase I periodontal treatment. The viability of cells was 0.86±0.13 OD/540 in healthy group and 0.4±0.25 OD/540 in periodontitis-affected group (P=0.035). The proliferation ability (population doubling time) of cells obtained from healthy teeth was 4.22±1.23 hours. This value was 2.3±0.35 hours for those obtained from periodontitis-affected teeth (P=0.02).

Conclusions: Viability and proliferation ability of cells isolated from the periodontium of healthy teeth were significantly greater than those of cells isolated from the periodontitis-affected teeth.

Keywords: Stem Cells; Periodontitis; Tooth; Regeneration

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INTRODUCTION

Stem cells (SCs) are unspecialized cells that are capable of self-renewal and have the potential to renew and maintain themselves for long periods of time and differentiate into different cell lineages with specialized functions [1,2]. Possible applications of SCs for repair and regeneration of damaged tissues are now the focus of attention of many researchers [3]. The autologous or allogeneic transplantation of SCs or their further differentiated progeny in vivo may be a potential therapeutic strategy, alone or in combination with the conventional treatments [4]. Basically, SCs can be isolated during embryogenesis (embryonic SCs) or from post-natal tissues (adult SCs) [5]. Recent evidence suggests that most tissues retain a population of resident SCs (adult SCs) throughout life [5].

Adult or somatic stem cells (ASCs) are now considered to be potentially suitable for tissue regeneration in humans; ASCs are undifferentiated cells isolated from a tissue with differentiated cells. They have the responsibility of maintaining and repairing their tissue of origin and are found in the brain, peripheral blood, skeletal muscle, cutaneous tissue, bone marrow, liver and blood vessels [5,6]. New experiments in dentistry have been designed to identify and characterize dental-derived SC populations for regenerative purposes [7-9]. Identification of SCs in a tissue is a difficult task and must be done based on indirect properties of these cells including surface protein repertoire expression, slow cell cycle, clonogenicity or their undifferentiated state. But, all these criteria are

non-specific. To show "stemness", assessment of self-renewal seems necessary. In this method, a putative SC (clonal analysis) is isolated and serially transplanted for long-term tissue reconstruction [7,9]. Ideally, ASCs can be isolated from a patient and after in vitro amplification and/or differentiation, they could be re-injected to the same patient; thus, avoiding immune rejection, as is the case for allografts or xenografts [10-12]. However, the ideal protocol for human pathologies has yet to be achieved.

Periodontal disease is a major public health problem and a common cause of tooth loss. The ultimate goal of periodontal treatment is to prevent progressive attachment loss and regenerate the periodontal supporting tissues. In order to achieve successful periodontal regeneration, new epithelial seal, connection tissue fibers inserted into the root structure, acellular cementum regenerated on the root surface and regaining of alveolar bone height are required [13]. Conventional periodontal therapy may effectively prevent disease progression, but bone needs to be removed in some cases to create better physiologic contours, and healing is almost always by repair; thus, the architecture or the function of the lost tissue would not be restored [14]. Bone autografts, allografts and cell occlusive barrier membranes are used for regeneration of the lost tooth structure. However, all these reconstructive procedures have limited predictability and efficacy [14].

With the advances in cellular and molecular biology, novel therapeutic strategies have been developed aiming at regeneration of injured or lost tissues [4,15]. Tissue engineering is a multidisciplinary field aiming to generate tissues and organs. It is based on identification of appropriate cells and their seeding on scaffolds. Morphogenic signals are also needed to induce their proliferation and differentiation into specific cell types [16,17]. Regeneration of lost periodontal tissue is an ultimate goal in periodontal therapy. The periodontal ligament (PDL) is a type of non-mineralized connective tissue that has long been recognized to contain a population of progenitor cells [18]. A previous study [18] identified a population of stem cells in human PDL, which were later found to be capable of differentiating into cementoblast-like cells, adipocytes and connective tissue rich in collagen type I in vitro and in vivo [18-21]. These cell types possess mesenchymal SC characteristics similar to bone marrow mesenchymal cells, such as colony forming ability, self-renewal and multilineage differentiation capabilities being able to differentiate into odontoblast-like or cementoblast/osteoblast-like cells, chondrocytes, adipocytes and neural-like cells [22,23].

The masticatory forces apply constant strain to PDL. Therefore, periodontal ligament stem cells (PDLSCs) may play an endogenous role in maintaining the number of cells in the PDL. This might explain why they are better than other dental SC populations at forming PDL-like structures [9]. Moreover, PDLSCs transplanted into immunocompromised mice and rats demonstrated the capacity of periodontal tissue regeneration and repair [24].

Considering the applications of human PDLSCs, this study sought to compare PDLSCs derived from healthy teeth with those derived from teeth with periodontitis.

MATERIALS AND METHODS

This study was approved by the Medical Ethics Committee of Hamadan University of Medical Sciences (#P/16/35/9/5464) and informed consent was obtained from the donors.

Stem cell isolation:

In this experimental study, PDLSCs were isolated from the healthy periodontium (group 1) around roots of five premolar teeth that were clinically sound and belonged to young individuals. The teeth had to be extracted for orthodontic purposes. The SCs of periodontitis-affected teeth were divided into two groups:

periodontally affected teeth with hopeless prognosis, which had undergone scaling and root planning one month earlier (group 2) and teeth with the same condition with no previous history of periodontal treatment of any kind (group 3).

Patients in group 1 were visited one week before tooth extraction and scaling and polishing were done if needed. Patients were advised to brush their teeth thoroughly. For extraction, prep and drape were performed and local anesthesia was administered. Risk of infection transmission was minimized by asking the patient to rinse diluted povidone iodine, using precisely sterilized pack of instruments and wearing sterile gloves, mask and gown. Extraction was done with minimal wobbling. After tooth extraction, the assistant held the coronal portion of root by sterile pliers and the surgeon separated the crown (in group 1) or a portion of root adjacent to the pocket wall (in groups 2 and 3) by a disc and high-speed handpiece under copious irrigation with saline solution to prevent over-heating and subsequent cell damage. Care was taken not to damage the cells by inadvertent movements of pliers. Another assistant opened a tube containing α-modified Eagle's medium (α-MEM) and then the root was 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 using wax sheet, immediately. The PDL tissues attached to the middle third of the root surface were removed using a surgical scalpel. Coronal and apical portions of the ligament were not used to avoid contamination with gingival and pulpal cells. The method of SC culture described by Gay et al, [25] was adopted as follows: PDL cells were scraped from premolars and transferred to a plate containing α-MEM and 15% fetal bovine serum (FBS) and were then enzymatically digested for one hour at 37°C in a solution of 3mg⁄mL collagenase type I. Then, the samples were centrifuged at 400g for 10 minutes and plats were expanded with α-MEM containing 15% FBS and 1% antibiotics in six-well plates and cultured at 37°C and 5% CO2. After four days of culture, numerous fibroblastoid cells migrated from the explants. At seven days, adherent cells, which were 80–90% confluent, were washed twice with phosphate-buffered saline and detached from the culture surface using 0.25% trypsin-EDTA solution (Gibco, Darmstadt, Germany) and plated in tissue culture polystyrene flasks at 5×103 cells/cm^2. Primary cultures of PDLSCs mainly consisted of colonies of bipolar fibroblastoid cells.

The methyl thiazol tetrazolium (MTT) assay was performed after third passage; 5×105 cells were cultured in each well of a 24-well plate. After seven days, viability of cells was evaluated using the MTT assay.

The MTT assay:

The MTT assay is based on metabolic activity of cells. In this method, yellow tetrazolium [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] is converted to purple insoluble formazan crystals by MTT succinate reductase of mitochondrial respiratory chain that is active only in living cells. To accomplish the MTT assay, the supernatant was replaced with 60 µL of MTT solution. Formazan crystals were formed after 1-1.5 hours of incubation at 37°C and 5% CO2. After dissolving crystals in 300 µL of dimethyl sulfoxide, the absorbance was read at 540-630nm wavelength using ELISA Reader. Then, the rate of cell proliferation in healthy and periodontitis-affected groups was assessed. After third passage, 105 cells from each group were cultured in 25cm flasks in Dulbecco's modified Eagle's medium containing 15% FBS for five days, trypsinized and counted. The cell population doubling time was calculated using the formula below [26]:

Population doubling number= (log N/NO × 3.31)

Where N is the amount of cells at the end of procedure and NO is the amount of cells at the beginning of culture.

Flow cytometric analysis:

A single cell suspension was prepared from the

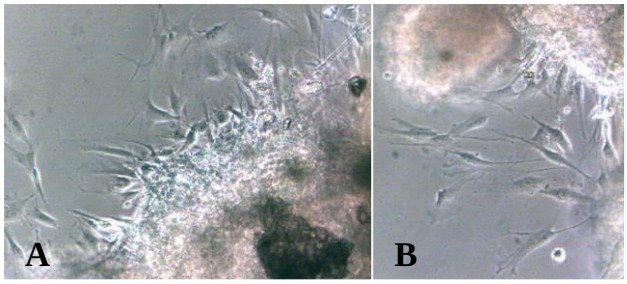

Fig. 1: Spindle-shaped cells after four days of culture at ×100 (A) and ×200 (B) magnifications

PDLSCs isolated from the PDL of healthy and periodontitis-affected teeth, which contained 1.0×106 cells in their third passage and subjected to digestion by trypsin/EDTA digestion. Cells were then fixed in 4% paraformaldehyde for 10 minutes. After re-suspension in 1% bovine serum albumin (ICN Biomedicals, Aurora, OH, USA) blocking buffer, the cells were incubated with primary CD105, CD146, CD90, CD45, CD31, CD34, CD106 and CD73 antibodies (experimental cells; 10 mg/mL) or phosphate-buffered saline (control cells) at 4°C for three hours, followed by secondary fluorescent antibody at room temperature for one hour. The percentage of CD105, CD146 and CD90 positive and CD45, CD31, CD34, CD106 and CD73 negative stained PDLSCs was measured using FACS Calibur™ flow cytometry (Becton Dickinson Immunocytometry Systems, CA, USA). For analysis of CD105, CD146 and CD90 positive and CD45, CD31, CD34, CD106 and CD73 negative cells, the percentage of cells positioned in the right side of the M1 gate was measured. The M1 gate was defined as the peak of negative control cells without primary antibody. The percentage of relatively CD105, CD146 and CD90 positive and CD45, CD31, CD34, CD106 and CD73 negative cells was calculated from the percentage of the experimental cells minus the percentage of the control cells. T-test was applied to compare the results of MTT assay and cell population doubling time between groups.

RESULTS

Cell culture:

As described earlier, three groups of PDLSCs were studied. No cells were obtained from the third group (periodontitis affected teeth that had not received phase I of periodontal treatment). But at four days, morphological observations in the remaining two groups revealed spindle shaped cells adherent to the bottom of the plates (Fig. 1).

Results of flow cytometry analysis:

Flow cytometry performed on the cells obtained from the two groups identified markers on these cells. As shown in Fig. 2, examined cells demonstrated CD105, CD146 and CD90 surface markers. In addition, these cells were negative for CD45, CD31, CD34, CD106 and CD73. This indicates that examined cells had characteristics similar to mesenchymal stem cells.

Results of MTT assay:

The optical density was 0.86±0.13 OD/540 in group 1 and 0.4 ±0.25 OD/540 in group 2. T-test reported a significant difference in this regard between the two groups (P=0.035). Comparison of the viability of the two groups is shown in Fig. 3.

Results of doubling of cell population:

The population doubling time of cell population was 4.22±1.23 hours in group 1 and 2.3±0.35 hours in group 2. T-test reported a significant difference between the two groups in this regard (P=0.02). Comparison of the proliferation ability of the two groups is demonstrated in Fig. 4.

DISCUSSION

Periodontitis is among the most common infectious diseases worldwide. Different treatment protocols, from non-surgical mechanical debridement to periodontal surgery and pharmaceutical therapy have been proposed for treatment of periodontal disease. However, none of these techniques can regenerate the recessed tissues due to periodontal disease.

Tissue engineering approaches have been mainly

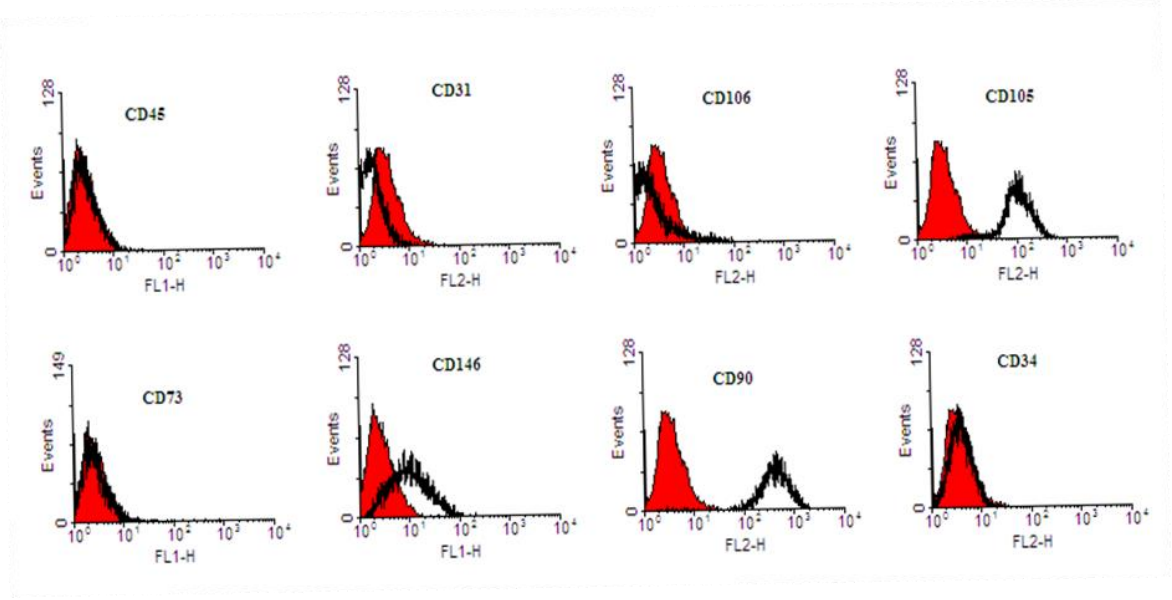

Fig.2: Flow cytometric analysis

investigated in vitro and have yet to become applicable in the clinical setting. Moreover, previous studies on SCs of dental origin have mainly obtained these cells from the lost tissues such as SCs obtained from the PDL, permanent and deciduous dental pulp, dental follicle, impacted third molars or healthy premolars extracted for orthodontic purposes [27]. These are not readily accessible sources in the clinical setting. A recent study reported isolation of SCs from dental pulp affected with pulpitis [28] and presence of PDLSCs in periodontitis affected teeth was also demonstrated by Park et al [29].

We investigated the viability and proliferation ability of PDLSCs obtained from teeth with periodontitis and hopeless prognosis, with the aim of using them for tissue regeneration, if they have efficient viability and proliferation ability. The results of our study showed that stem cells can be isolated from the periodontium of periodontitis-affected teeth that have undergone phase I of periodontal treatment (scaling and root planing). These cells can successfully proliferate as well. Periodontitis affected teeth that had undergone phase I of periodontal treatment contained PDLSCs and these cells were viable and had proliferation ability; however, these abilities were much less than those of cells obtained from healthy teeth periodontium. Isolation of stem cells from periodontitis-affected periodontium and proliferation ability of these cells (even at low levels) are promising findings in need of further investigation.

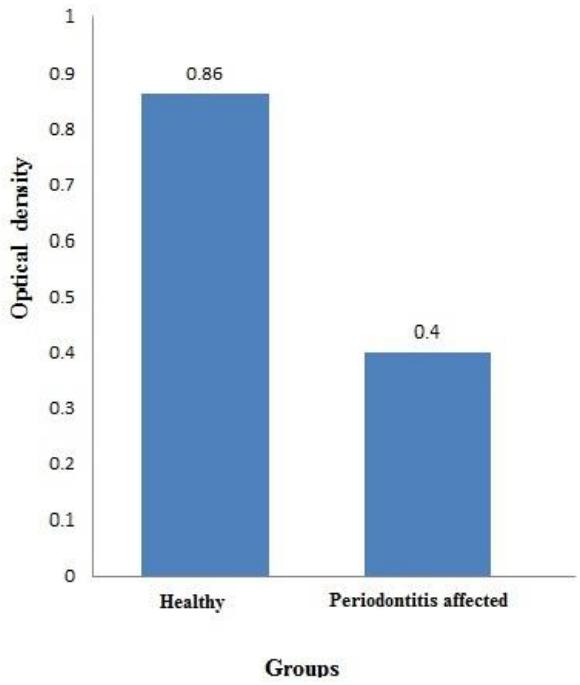

Fig. 3: Comparison of cell viability between the two groups (t-test)

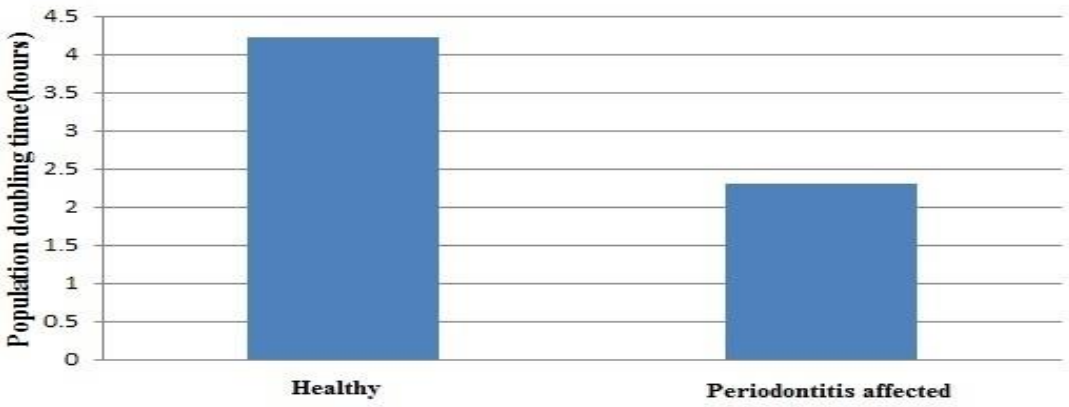

Fig. 4: Comparison of proliferation ability of cells between the two groups (t-test)

In the current study, we obtained cells from the middle third of the root surface, which was covered by bone and not affected by periodontitis; this area had no contact with the periodontal pocket wall. In a study by Park et al, [29] SCs were isolated from the granulation tissue around periodontitis affected teeth associated with bone loss. These cells had the characteristics of PDLSCs and their proliferation ability was the same as that of cells obtained from healthy periodontal tissue. They concluded that inflammation had no effect on proliferation ability of PDLSCs and the inflammation process and immune response enhanced the migration ability of PDLSCs. In the study by Chen et al, [30] on SCs, the PDLSC population increased on the periodontitis-affected cementum, in comparison with the healthy group. The results of these studies suggest that in inflamed areas, the inflammatory cells are recruited towards the inflammation site. As mentioned above, in our study, unlike the study by Park et al, [29] proliferation ability of cells obtained from the periodontitis affected teeth was less than that of cells obtained from the periodontium of healthy teeth. Such a difference in the results may be attributed to differences in cell origin; because we obtained cells from PDL tissues without bone resorption, while Park et al, [29] obtained cells from the granulation tissue of pocket wall around teeth with periodontitis and bone resorption. The cells of pocket wall are in a severely inflamed environment and have proliferated in response to inflammation; thus, the repair process has already initiated. In fact, destruction and repair simultaneously occur in periodontal pockets.

Cells isolated from deeper areas without bone loss (as in the middle third of the root surface chosen in our study) are viable but since the repair process has not been initiated here, these cells may not have proliferation ability as high as that of granulation tissue cells. Thus, we recommend isolating cells from deeper areas of periodontium not affected by disease in teeth with periodontitis. However, this issue is in need of further investigation and the current study was a preliminary one to pave the way for further research in this field of science.

CONCLUSION

Within the limitations of this study, we successfully isolated stem cells from the periodontium of both healthy and periodontitis-affected teeth that had undergone phase I of periodontal therapy. The viability of PDLSCs isolated from the periodontium of healthy teeth was higher than that of periodontitis affected teeth.

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