Autologous Dental Pulp Stem Cells in Regeneration of Defect Created in Canine Periodontal Tissue

Afshin Khorsand, DMD, MS
Mohamadreza Baghaban Eslaminejad, PhD
Mohadeseh Arabsolghar, DMD, MS
Mohijan Paknejad, DMD, MS
Baharak Ghaedi, DMD, MS
Amir Reza Rokn, DMD, MS
Neda Moslemi, DMD, MS
Hamid Nazarian, MSc
Shahrbanoo Jahangir, MSc

This study aimed to investigate effects of dental pulp stem cells (DPSCs) on regeneration of a defect experimentally created in the periodontium of a canine model. Surgically created mesial 3-walled periodontal defects with ligature-induced periodontitis were produced bilaterally in the first lower premolar teeth of 10 mongrel dogs. Simultaneously, DPSCs were derived from the maxillary premolar teeth of the same dogs. Four weeks after creation of the periodontitis model, autologous passaged-3 DPSCs combined with Bio-Oss were implanted on one side as the test group. On the other side, only Bio-Oss was implanted as a control. Eight weeks after surgery, regeneration of the periodontal defects was evaluated histologically and histomorphometrically in terms of bone, periodontal ligament (PDL), and cement formation. Histologically, in all test specimens (10 defects), regeneration of cementum, bone, and PDL was observed. In the control groups, although we observed the regeneration of bone in all defects, the formation of cementum was seen in 9 defects and PDL was seen in 8 defects. Histomorphometric analyses showed that the amount of regenerated cementum and PDL in the test groups (3.83 ± 1.32 mm and 3.30 ± 1.12 mm, respectively) was significantly higher than that of the control groups (2.42 ± 1.40 mm and 1.77 ± 1.27 mm, respectively; P < .05). A biocomplex consisting of DPSCs and Bio-Oss would be promising in regeneration of periodontal tissues.

Key Words: dental pulp stem cells, regeneration, periodontal defect, Bio-Oss

INTRODUCTION

Periodontitis is a complex disease of the periodontium that results in considerable defects to alveolar bone, gingival tissue, and periodontal ligament (PDL).1 Reconstruction of defects resulting from periodontal disease is a major challenge to regenerative medicine in the oromaxillofacial field. On the other hand, in the field of tissue regeneration, the use of tissue-resident stem cells as regenerative materials has gained considerable attention owing to their self-renewal property and multilineage differentiation capacity.

Among stem cells, dental stem cells are of interest as they are easily accessible and can be collected noninvasively with low morbidity.2 In this context, the periodontal ligament stem cell (PDLSC) has been successfully investigated in some animal models. Liu et al3 investigated the utility of autologous PDLSC to treat ligature-induced periodontitis in miniature swine. The 3-walled defect was generated in the first molar area of miniature
swine, and autologous PDLSCs obtained from extracted teeth were transplanted into the surgically created periodontal defect areas. Akizuki et al performed a pilot study in beagle canine teeth to investigate periodontal regeneration by use of PDLSC in dehiscence defects that were surgically created on the buccal surface of the canine first molar. Autologous periodontal ligament (PDL) cells obtained from extracted canine premolars were induced to form sheets when a temperature-responsive cell culture dish was used. The sheet with a reinforced hyaluronic acid carrier was then applied to the defects. In another study, Dogan et al researched PDL-derived stem cells for the regeneration of periodontal tissue in the canine model by seeding fibroblast-like cells derived from regenerated PDL in artificial furcation defects.

A few studies have researched the regenerative potential of dental pulp stem cells (DPSCs), which were first isolated by Gronthos et al. They described DPSCs as colonogenic cells that were capable of producing osteoblastic and chondrocytic cells in vitro and odontoblastic cells in vivo. The following investigations have indicated the broad differentiation capacity of the pulp-derived cells, which included their ability to give rise to neural and endothelial cell lineages. In addition, some studies have indicated that DPSCs are capable of forming well-vascularized lamellar bone after grafting. For example, de Mendonça Costa et al created 2 symmetric full-thickness cranial defects (5 × 8 mm in dimension) on the rat parietal region and attempted to reconstruct the defects with collagen membrane and DPSCs.

The aim of the present study is to examine the potential of DPSCs combined with biomaterials to promote regeneration of periodontium after experimentally created periodontitis is induced in canine models. Dogs are appropriate models because their periodontal tissues and tooth size are quite similar to those of humans. In addition, they often develop early dental plaque, which has many structural similarities to that occurring in humans. Furthermore, periodontal diseases can be easily induced in dogs. Although subgingival plaque formation in dogs may not develop identically to that in humans, dogs may still serve as a conventional model for investigation.

The bone regenerative capacity of DPSCs has been investigated previously, however, to date no investigation has evaluated DPSCs in the regeneration of periodontium that consisted of cementum, PDL, and supporting bone. The scaffold used in this study was xenograft, a natural bovine bone mineral with osteoconductive properties and high biocompatibility. This biomaterial has been tested in multiple randomized clinical trials and registered in the Cochrane Library.

**Materials and Methods**

**Animals**

Ten male mongrel dogs with healthy periodontium (1–2 years old, weighing 14–22 kg) were obtained from the Institute of Animal Science of Tehran 2 weeks before the beginning of the investigation. The dogs were kept under conventional conditions, vaccinated, and treated with antifungal drugs. Animal were fed soft food (Friskies, Purina, Marne La Vallee, France).

**Cells**

Based on our pilot study, we decided to extract 2 maxillary premolar teeth from each dog for DPSCs. Teeth were kept in Dulbecco’s modified eagle medium (DMEM, Gibco, Paisley, UK) and quickly sent to the laboratory for cell culture. At the Royan Institute Cell Culture laboratory, tooth surfaces were cut around the root-enamel boundary using dental fissure burs. Pulp tissues were then gently collected from the chambers and subjected to enzymatic digestion using a solution of 3 mg/mL collagenase type I (Sigma, St Louis, Mo) and 4 mg/mL dispase (Sigma) for 30 minutes at 37°C. The digest was provided with about 3 mL DMEM supplemented with 10% fetal bovine serum (FBS; Gibco) and centrifuged at 1200 rpm for 5 minutes. A single-cell suspension was then prepared and cells were plated at 10^3 cells/well in 6-well culture plates in an atmosphere of 5% CO₂ and 37°C. The culture medium was changed twice weekly until confluency was achieved. Confluent cultures were passaged at 1:3 ratios until sufficient cells became available for additional experimentation.

**Flow cytometry**

Flow cytometric analysis was used to characterize the isolated cells with respect to their surface antigen profile. About 1.5 × 10^5 passaged-3 cells were suspended in 100 µL of phosphate buffered saline (PBS) in 5 mL tubes that contained 5 µL of the following fluorescein isothiocyanate–conjugated
antibodies: CD146, CD44, CD90, SSEA-4, and anti-macrophage (Becton Dickinson, Franklin Lakes, NJ) followed by incubation at 4°C for 30 minutes in a dark room. The solution was then centrifuged at 1200 rpm for 4 minutes. The cells were dispersed in 300–500 μL washing buffer and then analyzed by flow cytometry (FACSCalibur cytometer equipped with 488 nm argon lasers, BD Bioscience, San Jose, Calif). The isotope controls were IgG 2 and IgG 1. WinMDI software (Version 2.9; Purdue University, West Lafayette, Ind) was used to analyze the flow cytometric results.

**Multilineage differentiation**

**Osteogenesis**

Passaged-3 cells were plated in 6-well culture plates until confluency. Then, the proliferation medium was replaced by induction medium that included DMEM supplemented with 50 μg/mL ascorbic acid 2-phosphate, 10 nm dexamethasone, and 10 μM beta glycerol phosphate (Sigma). The differentiation culture was maintained for 21 days, during which cultures were fed twice weekly. Alizarin red (Sigma) staining as well as reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was used to examine differentiation.

**Adipogenesis**

Passaged-3 cells were cultivated in 6-well culture plates until confluency. The medium was then substituted with differentiation medium that consisted of DMEM supplemented with 100 nm dexamethasone and 50 μg/mL indometacin (Sigma). After 21 days of incubation and twice-weekly medium replacement, differentiation was evaluated with oil red staining in addition to RT-PCR analysis.

**Chondrogenesis**

The micro-mass culture technique was used to promote chondrogenic differentiation of isolated cells. Passaged-3 cells were suspended in 5 mL DMEM medium in 15 mL tubes and centrifuged at 1200 rpm for 5 minutes. The supernatant was removed and chondrogenic medium was added onto the cell pellet. The chondrogenic medium consisted of DMEM supplemented with 3 ng transforming growth factor beta, 10 ng bone morphogenetic protein-6, 50 mg/mL transferrin-selenium-insulin, 50 mg bovine serum albumin, and 1% FBS (all from Gibco). The culture was maintained at 37°C and 5% CO₂ for 21 days, after which the culture was prepared for light microscopy. Sections 5 μm thick were created and stained with toluidine blue. The culture was analyzed by RT-PCR for expression of cartilage specific genes.

**RT-PCR analysis of gene expression**

Cellular differentiation potential was determined by RT-PCR detection of specific gene expression. Total RNA was collected from the cells induced to differentiate along bone, cartilage, and adipose cell lineages, as described previously, using RNX-Plus solution (CinnaGen Inc, Tehran, Iran). Before reverse transcription, RNA samples were treated with DNase I (Fermentas, Opelstrasse, Germany) to remove contaminating genomic DNA. The standard reverse-transcription reaction was performed with 5 μg total RNA using Oligo (dT) 18 as a primer and the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Opelstrasse, Germany) based on the manufacturer’s instructions. Reaction mixtures for polymerase chain reaction (PCR) included 2.5 μL cDNA, 1× PCR buffer (AMS, CinnaGen, Tehran, Iran), 200 μM dNTPs, 0.5 μM of each primer pair (Table 1) and 1 unit/25 μL reaction Taq DNA polymerase (Fermentas). Each PCR was performed in triplicate and under linear conditions. The products were analyzed on 2% agarose gel and visualized by ethidium bromide staining.

**Scaffold**

In this study Bio-Oss granules were purchased (Geistlich, Osteohealth Biomaterials, Bern, Switzerland) and used. Measurements of the crystalline size of Bio-Oss have shown the same characteristics as the tiny crystal size observed in clinically normal human bone. The spongiosa structure indicates an interconnected pore system (300–1500 μm) with a crystalline size of 10–60 nm.

**Cell loading**

To load the cells, 3 or 4 Bio-Oss granules were placed in small wells and 2 × 10⁷ autologous passaged-3 DPSCs were placed on the top surface of the scaffolds. The cultures were placed in an incubator at 37°C. At this time, the cells penetrated into the scaffold porosity and attached to the ceramic surfaces. Two hours after loading initiation, constructs were used for implantation. To calculate the amount of cells successfully loaded into the scaffold.
pores, all cells that appeared within the wells, either floating or adhered, were collected and counted with a hemocytometer. To ensure that DPSCs were within the scaffold surfaces, the loaded Bio-Oss were fixed in 10% formaldehyde in PBS buffer, decalcified in 10% ethylenediamine tetra-acetic acid for 24 hours, and processed for light microscopic observation.

**Creation of a periodontitis model**

To create periodontitis, the method of Liu et al. was used. In brief, dogs were anesthetized with intramuscular injections of a combination of ketamine (8 mg/kg) and diazepam (0.5 mg/kg). Then, a crestal incision was made from the mandibular canine to the midbuccal of the first premolar and the

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Primers used in reverse transcriptase-polymerase chain reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genes</td>
<td>Primer Sequences (5’-3’)</td>
</tr>
<tr>
<td>Col IA1</td>
<td>F: 5’ tca cct acc act gca aga ac 3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’ agt tta cag gaa gca gac agg 3’</td>
</tr>
<tr>
<td>COL II</td>
<td>F: 5’ caa gag cag cat tgc cta cc 3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’ agt tag ttt cct gcc tct gc 3’</td>
</tr>
<tr>
<td>LPL</td>
<td>F: 5’ ttg aac atg tgt ggg tat ctg</td>
</tr>
<tr>
<td></td>
<td>R: 5’ cta ggg cct tta ctg act gga</td>
</tr>
<tr>
<td>Aggrecan</td>
<td>F: 5’ aca gga ttg aag tca gtg gag 3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’ gtt gac aaa ctc ctc ttc ctc 3’</td>
</tr>
<tr>
<td>GAPDH</td>
<td>F: 5’ cca cgg caa att cca cgg cac ag 3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’ ggg gtc cct ccc atg cct cgc tc 3’</td>
</tr>
<tr>
<td>Osteopon</td>
<td>F: 5’ acg atg tag atg aag atg atg g 3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’ gct ttg act taa tgc gtc gac 3’</td>
</tr>
<tr>
<td>PPARG 2</td>
<td>F: 5’ atc cct cct cca tgc tgt tat g 3’</td>
</tr>
<tr>
<td></td>
<td>R: 5’ ata gtt tgg agt gga aat gct g 3’</td>
</tr>
</tbody>
</table>

**Figure 1.** Creation of periodontitis and implantation of biomaterial. (a) Photograph of surgical defect in the mesial portion of the dog’s lower premolar. (b) Ligature around the cervical portion of the tooth for the creation of periodontitis. (c) Second phase of surgery: Implantation of biomaterial was performed 4 weeks after creation of the model. (d) Suturing with 3-0 nylon thread.
A mucoperiosteal flap was elevated. Alveolar bone was removed by a surgical bur, and a surgical defect was created bilaterally in the mesial of the mandibular first premolar (Figure 1a). The surgical defect was 3 mm buccolingually, 5 mm apico-coronally, and 8 mm mesiodistally. The surface of the root was removed by a round bur to ensure removal of the cementum and PDL. Two notches, one at the level of the bone crest and the other on the floor of the defect, were made on the root surface by a round bur. In total, 20 defects were created in 10 dogs. A 3-0 silk thread was used around the cervical region of the first premolars to accelerate the accumulation of microbial plaque (Figure 1b).

**Transplantation**

Four weeks after creation of the model, signs of periodontitis, such as bleeding on probing, pocket formation, and loss of attachment, were observed. At this time under general anesthesia, the teeth were cleaned, the full-thickness flap was raised, and granulation tissue was removed from the defect by curettes. Then, scaling and root planning was performed to decontaminate the root surface. The
defects were randomly divided into 2 groups. On one side, about 3 or 4 Bio-Oss granules (1–2 mm) were implanted, whereas on the other side, the same amount of Bio-Oss granules loaded with autologous passaged-3 DPSCs were grafted (Figure 1c). The flap was advanced and sutured by the use of 3-0 nylon thread (Figure 1d). All animals received cefazolin (22 mg/kg, every 12 hours for 3 days) and tramadol (2 mg/kg, every 12 hours for 3 days) intramuscularly. A mouthwash of chlorhexidine digluconate was administered by a cotton roll twice daily for 2 weeks at the surgical sites.

**Histologic and morphometric evaluation**

Eight weeks after transplantation, all animals were killed by an overdose of sodium thiopental. Samples of the first premolars with mesial defects were harvested, fixed with 10% buffered formalin, decalcified in 15% nitric acid, and embedded in paraffin. About 9 sections were cut: the first section was cut...
at the mid-buccolingual aspect of the defects and then every section was cut bilateral from the first section at 5 μm thicknesses, stained with hematoxylin and eosin, and evaluated by light microscope (Olympus D 25, Olympus, Tokyo, Japan). Each section was analyzed quantitatively and histomorphometrically by analysis software (Olympus, version 3.2) according to the following parameters: (1) cementum formation, that is, the length of cementum formed between 2 notches; (2) alveolar bone formation, that is, the length of bone formed between 2 notches; and (3) PDL formation, that is, the length of PDL formed between 2 notches.

**Statistical analysis**

Groups were compared using the Wilcoxon signed ranks test. Statistical analysis was considered significant at $P < .05$.

**Results**

**Cell cultures**

The primary cultures established from dental pulp cells contained multiple colonies that consisted of fibroblastic cells (Figure 2a). A number of small clear cells were also present among the fibroblastic cells. The primary culture reached confluency in 10 days as the colonies grew (Figure 2b). In the subcultures cells tended to proliferate rapidly, reaching confluency in about 1 week.

**Flow cytometry**

According to flow cytometry results, most of the cells isolated from canine dental pulp tended to express CD90 and CD44 surface antigens. Other studied antigens, such as CD146, SSEA-4, and anti-macrophage, were expressed in very low percentages in the studied cells (Figure 2c through g).

**Multilineage differentiation**

Based on our regular observations of osteogenic cultures, some nodule-like structures appeared a few days after culture initiation and increased in number over time. The nodules were the foci of osteogenesis and tended to stain heavily with alizarin red, which specifically stains mineralized matrix (Figure 3a). The RT-PCR analysis revealed that bone-related genes, including osteopontin and CollA1, were expressed in these cultures (Figure 3b). In the adipogenic cultures, a few cells were observed that developed lipid droplets in their cytoplasm. These lipid droplets stained red with Oil Red O stain (Figure 3c). Furthermore, based on the RT-PCR results, adipose related-genes, such as lipoprotein lipase and peroxisome proliferators, activated receptor-gamma expressed in the differentiated cells (Figure 3d). Positive toluidine blue staining of the sections prepared from chondrogenic pellets demonstrated the production of metachromatic matrix at the chondrogenic cultures (Figure 3e). Chondrogenesis by dental pulp cells was further analyzed and confirmed by RT-PCR. According to these analyses, cartilage-specific genes, including aggrecan and collagen II, were expressed in the chondrogenic cultures (Figure 3f).

**Scaffolds**

Observation of the sections prepared from decalcified Bio-Oss loaded with DPSCs indicated that DPSCs were successfully loaded into the biomaterial internal pores (Figure 4). According to our results, about 60% of the loaded cells were successfully trapped within the scaffold pore systems.

**Clinical evaluation**

Suppuration, infection, gingival recession, and root exposure were not observed. There was no difference between the test and control groups during the 8-week healing period. Initial inflammation was comparable in both groups.
Histologic evaluation

In most histologic sections, Bio-Oss particles were found in both groups and observed to be surrounded by woven bone. Nevertheless, in 2 specimens from the control group a connective tissue capsule was seen around the bone graft particle (Figure 5a and b). The overall amount of residual particles appeared to be similar in both groups. Two specimens from the control group had relatively more residual particles. In all test groups and most of the control groups, epithelium migration stopped at the coronal portion of the coronal notch, but in 2 control groups, down growth of the epithelium was seen in the apical area of the coronal notch (Figure 5c and d).

Formation of new cementum, PDL, and bone were observed in all test specimens. In the controls, new bone was observed in all groups but the formation of cementum was seen in 9 defects and PDL was seen in 8 defects (Figure 5e and f). Regenerated cementum in the test group was thicker than that in the control and covered a larger surface of the root. However, in the control groups, the cementum tended to be smaller, separated, and scattered. According to our findings, dentinoid (dentin-like tissue) formed in only one test group.

Histomorphometric evaluation

There was no significant difference in bone formation between the test and control groups. The amount of bone formation in these groups was...
3.60 ± 1.06 mm in the test groups and 3.10 ± 0.82 mm in the control groups (Figure 6). New cementum formation in the test groups was 3.83 ± 1.32 mm compared to 2.42 ± 1.40 mm in the control groups (Figure 6), which was statistically significant (P < .05). Regarding PDL formation, the mean value was 3.30 ± 1.12 mm for the test groups and 1.77 ± 1.27 mm for the control groups (P < .05; Figure 6).

**DISCUSSION**

At present, the clinical results of periodontitis treatments are unsatisfactory. For this reason, new therapies such as stem cell therapy have gained considerable attention. DPSCs are adult stem cells that have several advantages over other types of stem cells, including ease of accessibility and noninvasive collection with low morbidity. Two groups of studies have examined the capability of DPSC for forming hard tissue. One group believes DPSCs, like bone marrow stem cells, differentiate into osteoblasts and can produce bone. The other group has shown that DPSCs differentiate into odontoblasts and are able to generate dentin-like tissue. To date, no investigations have evaluated the potential of DPSCs in regeneration of defects in periodontal tissues that consist of alveolar bone, cementum, and PDL. In the present study, however, this was investigated. According to our findings, implantation of DPSCs combined with Bio-Oss granules resulted in significant reconstruction of cementum and PDL in addition to regeneration of alveolar bone.

In the present study, DPSCs were isolated from maxillary premolar teeth and characterized before transplantation in terms of some surface antigens. We used CD146 and anti-macrophage marker to exclude the endothelial origin and macrophage nature of the cells, respectively. SSEA-4 is an embryonic stem cell marker used to examine pluripotency of the isolated cells, and CD90 and CD44 are mesenchymal stem cell (MSC) markers used to ensure that the cells were from the MSC population. The isolated cells possessed a tripotent differentiation potential capable of producing bone, cartilage, and adipose cells. Moreover, they grew adherent throughout the culture period. These characteristics were in agreement with properties proposed for MSCs by the Tissue Stem Cell Committee of the International Society for Cell Therapy. Therefore, the isolated cells in this study can be considered an MSC-like population with a dental pulp origin.

According to our observations, woven bone formed at the implantation site. This differed from former studies in which lamellar bone formation was reported after transplantation of DPSCs combined with collagen in rat calvaria. This difference might be due to the difference in animal (dog versus rat) and biomaterial (Bio-Oss versus collagen) types that were used in the two studies. The osteogenic effects of the DPSCs-collagen biocomplex have also been mentioned by d’Aquino et al in a human mandible defect. According to their findings, the samples were made up of well-organized and well-vascularized bone with a lamellar architecture that surrounded the haversian channels.

In the area of cementogenesis and PDL formation, the present study is the first to evaluate the potential of DPSCs. However, regeneration of cementum and PDL with other dental stem cells, particularly PDLSCs, has already been investigated. Akizuki et al investigated periodontal regeneration by use of PDLSCs in defects surgically created on the buccal surface of canine molars. In this study they created a dehiscence defect compared to the 3-walled defects of the current study, which are more similar to periodontal defects. Liu et al explored the potential of PDLSCs to treat periodontal defects in a porcine model of periodontitis. They showed that PDLSCs are capable of regenerating periodontal tissues. Liu et al quantified bone formation but not cementum and PDL formation, whereas in the present study, we quantified all tissue components that make up the periodontium. A study by Dogan et al is another example of the use of PDL-derived stem cells in the regeneration of periodontal tissue in canine models. This was a pilot study where a furcation defect was created in just one dog; however, in our study 10 dogs were used to assess DPSCs in 3-walled defects.

All the aforementioned cell-seeding studies may be useful and promising; however, numerous questions have been posed. It is recommended that researchers perform other studies that use another type of control group, such as autografts or other biomaterials that can be loaded with the growth factors. Furthermore, at this time there are...
numerous additional ways to examine regeneration and prove that the tissues produced are of the desired type, such as immunohistochemistry to identify tissue-specific antigens, in situ hybridization to demonstrate tissue-specific gene expression, micro-computed tomography to demonstrate the regeneration of mineralized tissue, and bone density measurements to analyze calcification of regenerated tissues. Without a broader range of experimental analyses the results are limited to very descriptive assessments of what the new tissues look like, which is not sufficient to demonstrate what the tissues actually are.

**CONCLUSION**

This investigation is the first study showing that DPSCs are capable of promoting periodontal regeneration. Along with a Bio-Oss scaffold, DPSCs would be an excellent biocomplex. In fact, although DPSCs seem to be a promising therapy in the treatment of periodontal diseases, additional research is needed in this area.

**ABBREVIATIONS**

DMEM: Dulbecco’s modified eagle medium  
DPSC: dental pulp stem cell  
FBS: fetal bovine serum  
MSC: mesenchymal stem cell  
PBS: phosphate buffered saline  
PDL: periodontal ligament  
PDLSC: periodontal ligament stem cell  
RT-PCR: reverse transcriptase-polymerase chain reaction

**REFERENCES**

5. Dogan A, Ozdemir A, Kubar A, Oygür T. Assessment of periodontal healing by seeding of fibroblast-like cells derived from...


