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

Running title: DPSCs in regeneration of periodontal tissue

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Abstract

Objective: This study aimed to investigate effects of dental pulp stem cells (DPSCs) on regeneration of the defect experimentally-created in periodontium of canine model. **Study design**: Surgically-created mesial three-walled periodontal defects with ligature-induced periodontitis were produced bilaterally in the first lower premolar teeth of ten mongrel canines. Simultaneously, DPSCs were derived from maxillary premolar teeth of the same animals. Four weeks after creation of the periodontitis model, on one side, autologous passaged-3 DPSCs combined with Bio-oss were implanted 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 both histologically and histomorphometrically in terms of bone, periodontal ligament (PDL) and cement formation. **Results**: Histologically, in all test specimens (ten 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 nine defects and PDL was seen in eight defects. Histomorphometric analyses showed that the amount of regenerated cementum and PDL in the test groups (3.83±1.32 and 3.30±1.12 mm, respectively) was significantly higher than the control groups (2.42±1.40 and 1.77±1.27 mm, respectively; $P<0.05$). **Conclusion**: Biocomplex consisting of DPSCs and Bio-oss would be promising in regeneration of periodontal tissues.
Keywords: 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 oromaxillo-facial 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 as well as multilineage differentiation capacity.

Among stem cells, dental stem cells are of interest since they are easily accessible and can be collected noninvasively with low morbidity[2]. In this context, the periodontal ligament stem cell (PDLSC) has previously been successfully investigated in some

animal models. Liu et al. investigated the utility of autologous PDLSC to treat ligature-induced periodontitis in miniature swine. The three-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[3]. Akizuki performed a pilot study in beagle canines 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 PDL cells obtained from extracted canine premolars were induced to form sheets when a temperature-responsive cell culture dish was used. Then, the sheet with a reinforced hyaluronic acid carrier was applied to the defects[4]. 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[5].

Despite PDLSCs, there were a few studies that researched the regenerative potential of dental pulp stem cells (DPSCs). DPSCs were first isolated by Gronthos et al. who described them as colonogenic cells that were capable of producing osteoblastic and chondrocytic cells in vitro and odontoblastic cells in vivo[6]. 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[6-9]. In addition, some studies have indicated the capability of DPSCs to form well-vascularized lamellar bone after grafting[2, 9-10]. For example, de Mendonça Costa et al. created two 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[11].

The aim of the present study is to examine the potential of DPSCs combined with biomaterials in order to promote regeneration of periodontium following experimentally-created periodontitis in canine models. Canines are appropriate models since the periodontal tissues and the size of teeth in canines are quite similar to those of humans. In addition, they have early dental plaque that has many structural similarities with that occurring in humans. Furthermore, periodontal diseases can easily be induced in this animal[12-16]. Although subgingival plaque formation in the canine may not develop identically to that in humans, the canine may still serve as a conventional model for investigation.

Until now, the bone regenerative capacity of DPSCs has been investigated[17-19], however 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 (NBM) with osteoconductive properties and high biocompatibility. This biomaterial has been tested in multiple randomized clinical trials and registered in the Cochrane Library[20].

Materials and Methods

Animals

Ten male mongrel canines with healthy periodontium (1-2 years old, weighing 14-22 kg) were obtained from the Institute of Animal Science of Tehran two weeks before the beginning of the investigation. Canines 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 two maxillary premolar teeth from each canine for DPSCs. Teeth were kept in Dulbeccos modified eagle medium (DMEM; Gibco, Paisley, UK) and quickly sent to the laboratory for cell culture. At the Royan Institute Cell Culture lab, 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, USA) 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 six-well culture plates, in an atmosphere of 5% CO_2 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 PBS in 5 ml tubes that contained 5 µl of the following FITC-conjugated antibodies: CD146, CD44, CD90, SSEA-4 and anti-macrophage (Becton Dickenson, Franklin Lakes, NJ, USA) followed by incubation at 4°C for 30 minutes in a dark room. The solution was then centrifuged at 1200 rpm for 4 minutes, cells were dispersed in 300-500 µl washing buffer and then analyzed by flow cytometry (FACS Calibur cytometer equipped with 488 nm argon lasers). IgG 2 and IgG 1 were isotope controls. WinMDI software was used to analyze the flow cytometric results.

Multilineage differentiation

Osteogenesis

Passaged-3 cells were plated in six-well culture plates until confluency. Then, the proliferation medium was replaced by induction medium that included DMEM supplemented with 50 µg/ml ascorbic2-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 RT-PCR analysis was used to examine differentiation.

Adipogenesis

Passaged-3 cells were cultivated in six-well culture plates until confluency. Then, the medium was 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 on to the cell pellet. Chondrogenic medium consisted of DMEM supplemented with 3 ng transforming growth factor beta, 10 ng bone

morphogenetic protein-6 (BMP-6), 50 mg/ml transferrin-selenium-insulin, 50 mg bovine serum albumin and 1% FBS (all from Gibco). The culture was maintained at 37ºC and 5% CO_2 for 21 days after which the culture was prepared for light microscopy. Five micrometer thick sections were created and stained with toluidine blue. The culture was analyzed by RT-PCR for expression of cartilage specific genes.

Reverse transcriptase-polymerase chain reaction (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 above, using RNX-Plus™. solution (CinnaGen Inc., Tehran, Iran). Prior to 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 based on the manufacturer's instructions. Reaction mixtures for PCR included 2.5 µl cDNA, 1x PCR buffer (AMS), 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 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, 3-4 Bio Oss granules were placed in small wells and 2×10^7 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% EDTA for 24 hours, and processed for light microscopic observation.

Creation of a periodontitis model

To create periodontitis, the method by Liu et al. was used[3]. In brief, canines 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 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 (Fig. 1A). The surgical defect was 3 mm bucco-lingually, 5 mm apico-coronally, and 8 mm mesio-distally. 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 canines. A 3-0 silk thread was used around the cervical region of the first premolars to accelerate the accumulation of microbial plaque (Fig. 1 B).

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 removed from the defect by curettes. Then, scaling and root planning was performed to decontaminate the root surface. The defects were randomly divided into two groups. On one side, about 3-4 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 (Fig. 1C). The flap was advanced and sutured by the

use of 3-0 nylon thread (Fig. 1 D). 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 two weeks at the surgical sites.

Histological and morphometrical evaluation

Eight weeks after transplantation, all animals were sacrificed 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 nine sections were cut: the first section was cut at the mid bucco-lingual of the defects and then every section was cut bilateral from the first section at 5 μm thicknesses, stained with H&E and evaluated by light microscope (Olympus D 25, Tokyo, Japan). Each section was analyzed quantitatively and histomorphometrically by analysis software (Olympus Company) according to the following parameters: 1) cementum formation: length of cementum formed between two notches; 2) alveolar bone formation: length of bone formed between two notches; 3) PDL formation: length of PDL formed between two notches.

Statistical analysis

Groups were compared using the Wilcoxon signed ranks test. Statistical analysis was considered significant at $p<0.05$.

Results

Cell cultures

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

Flow cytometry

According to flow cytometry results, the majority of 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 (Figs. 2C-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 (Fig. 3A). RT-PCR analysis revealed that bone-related genes, including osteopontin and ColIA1, were expressed in these cultures (Fig. 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 (Fig. 3C). Furthermore, based on the RT-PCR results, adipose related-genes such as LPL (lipoprotein lipase) and PPAR-gamma (peroxisome proliferators activated receptor-gamma) expressed in the differentiated cells (Fig. 3D). Positive toluidine blue staining of the sections prepared from chondrogenic pellets demonstrated the production of metachromatic matrix at the chondrogenic cultures (Fig. 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 (Fig. 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 (Fig. 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 eight week healing period. Initial inflammation was comparable in both groups.

Histological evaluation

In most histological sections Bio-oss particles were found in both groups and observed to be surrounded by woven bone. Nevertheless, in two specimens from the control group a connective tissue capsule was seen around the bone graft particle (Figs. 5A-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 two control groups down growth of the epithelium was seen in the apical area of the coronal notch (Figs. 5C-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 nine defects and PDL was seen in eight defects (Figs. 5E-F). Regenerated cementum in the test group was thicker compared to 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.

Histomorphometrical 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 (Fig. 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 (Fig. 6), which was statistically significant ($p<0.05$). Regarding PDL formation, the mean value for the test groups was 3.30±1.12 mm and the control groups was 1.77±1.27 mm ($p<0.05$; Fig. 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 possess several advantages over other types of stem

cells, including ease of accessibility and noninvasive collection with low morbidity. There are two groups of studies about the hard tissue formation capability of DPSC. One group believes that DPSCs, like bone marrow stem cells, differentiate into osteoblasts and can produce bone [2, 4, 11]. The other group has shown that DPSCs differentiate into odontoblasts and are able to generate dentin-like tissue [6, 21- 23]. There has been no investigation to evaluate the potential of DPSCs in regeneration of defects in periodontal tissues that consisted of alveolar bone, cementum and PDL. In the present study, this was investigated. According to our findings, implantation of DPSCs combined with Bio-Oss granules resulted in significant reconstruction of both cementum and PDL in addition to the regeneration of alveolar bone.

In the present study, DPSCs were isolated from maxillary premolar teeth and characterized prior to transplantation in terms of some surface antigens. CD146 and anti-macrophage marker were used 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. CD90 and CD44 are MSCs 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 [24]. 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 have been reported following transplantation of DPSCs combined with collagen in rat calvaria [11]. This difference might be due to the difference in animal (canine versus rat) as well as biomaterial (Bio-Oss versus collagen) types that have been used in the two studies. Well-osteogenic effects of the DPSCs-collagen biocomplex have also been mentioned by 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 [2].

In the area of cementogenesis and PDL formation, the present study is the first one to evaluate the potential of DPSCs. However, regeneration of cementum and PDL with other dental stem cells, particularly PDL-derived stem cells (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 [4]. 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. have explored the potential of PDLSCs to treat periodontal defects in a porcine model of periodontitis. They have shown that PDLSCs were capable of regenerating periodontal tissues [3.] In this study they quantified bone formation but not cementum and PDL formation, whereas in the present study we quantified all tissue components that comprised periodontium. A study by Dogan et al. was another example of PDL-derived stem cells in regeneration of periodontal tissue in canine models [5]. This was a pilot study where a furcation defect was created in just one canine, however in our study 10 canines were used to assess DPSCs in 3-walled defects.

All the above cell-seeding studies may be useful and promising; however numerous questions have been posed. It is recommended to 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, microCT 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 are not sufficient to demonstrate what the tissues actually are.

Conclusion

This investigation is the first study showing that DPSCs are able to promote periodontal regeneration. DPSCs along with a Bio-oss scaffold 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.

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Figure Legends

Fig. 1: Creation of periodontitis and implantation of biomaterial. A) Photograph of surgical defect in the mesial of the canine’s lower premolar. B) Ligature around cervical portion of tooth

for 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.

Fig. 2: Pulp-derived cell culture. A) A large colony formed at the primary culture, day 5. Bar= 200 µm, original magnification: 10x. B) Confluent culture of pulp derived cell culture. Bar=200 µm, original magnification: 50x. C-G) Flow cytometric analysis of passaged-3 canine pulp derived stem cells. The majority of the cells expressed CD90 and CD44 surface antigens. CD146, SSEA-4 and anti-macrophage were expressed on a few percentages of the cells.

Fig. 3: Multilineage differentiation potential of canine pulp-derived stem cells. Passaged-3 cells tended to differentiate along bone (A-B, bar=50 µm), adipose (C-D, bar=50 µm) and cartilage (E-F, bar=200 µm) cell lineages. Bar= 50 µm.

Fig. 4: Photomicrograph of section prepared from decalcified Bio-oss loaded with DPSCs. The inset indicates the rectangular area at a higher magnification. Bio-oss internal pores loaded with DPSCs original magnification: 10x, Bar = 250 µm.

Fig. 5: Histologic section from the repair tissue. (A) In some sections from the control, a connective tissue capsule(c) was seen around the bone graft particle (b). Bar=50 µm, original magnification: 50x. (B) In the majority of sections Bio-oss particles were surrounded by woven bone (w). Test group. Bar=50 µm, original magnification: 50x. (C) epithelium migration stopped at the coronal portion of the coronal notch. Test group. Bar=100 µm, original magnification: 10x. (D) Down growth of epithelium was seen in the apical portion of the coronal notch. Control group. Bar=100 µm, original magnification: 10x. (E) A sample of control group containing granulation tissue (g) without any formation of bone, cementum and PDL. Bar=100 µm, original magnification: 10x. (F) A sample of test group showing perpendicular fibers of PDL (p) that formed between bone (b) and cementum (c). Bar=100 µm, original magnification: 25x.

Fig.6: Histomorphometric data of amount of new bone, cementum and periodontal ligament formation in the test and control groups.

Table 1: Primers used in RT-PCR.