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The combined use of cell sheet fragments of periodontal ligament stem cells and platelet-rich fibrin granules for avulsed tooth reimplantation

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ABSTRACT

The aim of this study was to construct a cell transplant method consisting of cell sheet fragments of periodontal ligament stem cells (PDLSCs) and platelet-rich fibrin (PRF) granules to enhance periodontal healing in avulsed tooth reimplantation. To test this concept *in vitro*, human PDLSCs were isolated and characterized by colony forming unit assay, cell surface marker characterizations, and their osteogenic/adipogenic differentiation potential. The biological effects of autologous PRF as a growth factor-enriched endogenous scaffold on human PDLSCs were then investigated and quantified for statistical analyses, including cell viability and proliferation, alkaline phosphatase (ALP) activity, and the gene expression of bone sialoprotein (BSP), osteocalcin (OCN), collagen I (Col-I), and cementum protein 23 (CP23). It was found that the PRF induced a significant and continuous stimulation of proliferation in human PDLSCs throughout the 7-day incubation period. Furthermore, the PRF suppressed the osteoblastic differentiation of PDLSCs by decreasing ALP activity and the gene expression of BSP and OCN while up-regulating the mRNA expression levels of Col-I and CP23 during the testing period. To assess the potential application of the PDLSCs/PRF construct in tooth reimplantation, 36 incisors were extracted from 6 dogs. The incisors then underwent 2 h of dry storage and were randomly divided into four groups receiving different strategies of reimplantation, where the avulsed teeth were reimplanted with the use of the autologous PDLSCs/PRF construct (cell sheet fragments in combination with PRF granules), with the use of autologous PDLSCs or PRF alone, or without adjuvant use of PRF or PDLSCs. Eight weeks post-reimplantation, the PDLSCs/PRF group achieved a more effective periodontal healing, characterized by the regeneration of PDL-like tissues and a reduction of ankylosis and inflammation, compared with the other testing groups. These overall results suggest that the PDLSCs/PRF construct may be a useful tool for alveolar surgery that has the potential to improve the clinical outcomes in future avulsed tooth reimplantations.

1. Introduction

Millions of teeth are accidentally avulsed each year due to trauma in sports, motor vehicle accidents, criminal assaults, and fist fights, among other incidents. Tooth avulsion, defined as the complete displacement of the tooth from its original alveolar socket, is one of the most severe types of dental trauma [1]. Generally, tooth reimplantation is an effective therapy for tooth avulsion, provided that the patient acts quickly (e.g., within 2 h) and that the avulsed tooth is preserved in an appropriate medium, such as milk or physiological saline, because the viable cells in the remaining periodontal ligament (PDL) on the root surface play a crucial role in ensuring correct periodontal healing following avulsed tooth reimplantation [2,3]. Unfortunately, in most, if not all, cases, knocked-out teeth have been either out of the alveolar socket for a prolonged period of time or kept under non-physiological

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conditions, which increases the risk of PDL cell necrosis and, hence, results in ankylosis and replacement resorption of the tooth root post-reimplantation [2]. Of note, periodontal healing cannot be achieved for a very large majority of knocked-out teeth, resulting in rapid root resorption or, more frequently, dental ankylosis with subsequent bone substitution and, finally, tooth loss over time. Upper anterior teeth are more likely to suffer from trauma, and their loss can result in significant esthetic and functional problems. Therefore, there is an increased demand for more effective management of this condition [4].

Knowing that the major reason for tooth reimplantation failure is due to the damage of the stem cells remaining on the avulsed tooth, it is logical to postulate that the adjuvant use of outside cultured stem cells may improve the long-term prognosis and survival of these teeth. To identify a suitable cell population toward this goal, Dangaria et al. (2011) assessed the suitability of 3 odontogenic progenitor populations from dental pulp, PDL, and dental follicles for periodontal regeneration when exposed to natural and synthetic apatite surface topographies. These authors found that PDL progenitors featured elevated levels of periostin and scleraxis expression, an increased adipogenic and osteogenic differentiation potential, and pronounced elongated cell shapes on barren root chips when compared with dental pulp and dental follicle cells, indicating that the periodontal progenitor cell type plays crucial roles in the regeneration of true periodontal anchorage [5]. Although bone marrow mononuclear cells have also been used to enhance the periodontal healing of reimplanted dog teeth [6], there is mounting evidence that PDL-derived stem/progenitor cells are the best cell choice for periodontal tissue-engineering [7–10]. In addition, the use of PDL cells involves less religious and ethical concerns than using stem cells derived from bone marrow because they are easily obtainable from medical waste, i.e., teeth extracted for orthodontic, impaction, or irreversible periodontic reasons, thus facilitating translational research and future clinical applications in periodontal clinical therapies [8,11].

When considering other adjuvants for improving overall clinical outcomes, platelets activated with thrombin, such as platelet-rich plasma (PRP), have been demonstrated to play an important role in the healing of tissues after tooth reimplantation [6,12]. The efficiency of this process lies in the local and continuous delivery of a wide range of growth factors and proteins, mimicking the needs of physiological wound healing and reparative tissue processes [13]. Fortunately, researchers have begun to combine calcium chloride-activated PRP (PRP/Ca) and outside expanded cells (bone marrow mononuclear cells) for tooth reimplantation in animal models, resulting in larger areas of replacement resorption compared with the PRP/Ca only group, characterized by osseous growth into cementum with intense immunomarcation for tartrate-resistant acid phosphatase [6]. However, as suggested by the authors, additional studies are necessary to redesign the blood-derived materials based on tissue-engineering concepts because the PRP/Ca did not present an appropriate scaffold for undifferentiated cells during the treatment of avulsed teeth.

It has been known for decades that regenerative medicine using tissue-engineering-based constructs to enhance periodontal repair is a promising approach for the restoration of the structure and function of reimplanted teeth [5,6,12]. Considering the specific scaffold requirements for delivering and sustaining therapeutic stem cells on the root surface, it is clearly difficult for traditional transplants to be implanted into the space between the alveolar bone and root cementum, which ranges from 0.15 to 0.38 mm wide. Platelet-rich fibrin (PRF) has been referred to as the second-generation platelet concentrate, which has been demonstrated to have several advantages over traditionally prepared PRP, such as ease of preparation and a lack of the biochemical handling of blood [13,14]. Most importantly, the three-dimensional (3D) structure of PRF is optimal for delivering therapeutic cells to a specific site of injury [15–18]. Although the use of either platelet-rich preparations or periodontal ligament stem cells (PDLSCs) has been alone tested for tooth reimplantation, a combination of both interventions may offer best opportunity for beneficial clinical outcomes.

In this article, we hypothesized that a cell transplant consisting of cell sheet fragments of PDLSCs and PRF granules might be able to regenerate PDL tissues around the reimplanted teeth. To evaluate this newly designed cell transplant method, the biological effects of PRF on the behavior of human PDLSCs were initially investigated *in vitro*. For application in animal models of tooth reimplantation, dog PDLSCs were conducted into cell sheets to enhance the density of the final seed cells, and autologous PRF membrane was used as a growth factor-rich scaffold that may facilitate the immobilization of the cells on the surface of the root surface. This cell transplant method, based on tissue-engineering concepts, may open up new opportunities for the clinical management of periodontally involved hopeless avulsed teeth.

2. Materials and methods

2.1. Isolation and characterization of PDLSCs

2.1.1. Isolation of PDLSCs

Human PDLSCs were isolated and cultured according to previously reported protocols with slight modifications [11,19,20]. Briefly, human impacted third molars were extracted from 3 systemically healthy adults (23–28 years of age) at the School of Stomatology, Fourth Military Medical University. The teeth were immediately immersed into an ice-cold phosphate-buffered saline (PBS; Hyclone, Road Logan, UT, USA) solution that contained 100 U/mL penicillin/streptomycin (Sigma–Aldrich, St. Louis, MO, USA) for transfering to the laboratory. The PDL tissues in the middle third of the root surface were separated by blade, washed several times with PBS, and then minced into small cubes (1 mm^2). The tissue cubes were digested with 2 mL of α-minimum essential medium (α-MEM, Hyclone) containing 3 mg/mL collagenase (type I) and 4 mg/mL dispase (both from Sigma–Aldrich) for 15 min in a cell incubator. The digested PDL tissues were then transfered into 2 six-well plates (Nunc, Thermo, Denmark), covered with sterile coverslips, and cultured in α-MEM supplemented with 10% fetal bovine serum (FBS), 0.292 mg/mL glutamine (both from Hyclone), 100 U/mL/mL penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 until the cells successfully grew out from the PDL tissue pieces. The primary cells were then subcultured using a limiting dilution technique to obtain passage 0 single cell-derived clones (P0). Cell cultures at passages P3-P5 were used for the following *in vitro* study.

2.1.2. Colony forming assay

The human PDLSCs (P3) were plated into 90-mm dishes (two dishes for each cell line) at a density of 1×10^3 cells/well and cultured in complete medium for 12 days for the colony forming unit fibroblast (CFU-F) assays. The cells were fixed with 4% paraformaldehyde and then stained with 0.1% toluidine blue for 15 min. The dishes were then washed twice with distilled water, and aggregates containing more than 50 cells were scored as colonies. The aggregates containing less than 50 cells were excluded.

2.1.3. Flow cytometric analysis of cell surface markers

Human PDLSCs (P3) were used for a flow cytometry analysis of cell surface markers using a previously reported procedure [11,19]. After being digested with 0.25% trypsogen (Hyclone) and washed with PBS for three times, the cells were adjusted to a concentration of 1×10^6 cells/mL. Then, 100 μL of cell suspension was added to a microtube (Axygen, Union City, USA); each cell line were transferred into 5 tubes that were used for the identification of different cell markers. Subsequently, the following antibodies were added to the microtubes according to the manufacturer's instructions: STRO-1 (Biolegend, San Diego, USA), CD29 (eBioscience, San Diego, CA, USA), CD34 (Biolegend), and CD45 (Epitomics, Burlingame, CA, USA). Cell suspensions without added antibodies served as controls. All of the microtubes were incubated for 1 h at room temperature in the dark and then washed with PBS for three times. Finally, 300 μL of PBS supplemented with 3% FBS was added to the tubes, and the cells were then resuspended for analysis by using a flow cytometry cell sorting Vantage Cell Sorter (Becton & Dickinson, Mountain View, USA). The obtained data were analyzed using the Mod-Fit 2.0 cell cycle analysis program (Becton & Dickinson).

2.1.4. Osteogenic/adipogenic differentiation of PDLSCs

The human PDLSCs (P3) were cultured and induced to assess their mineral nodules and lipid formation *in vitro* (each cell line for four wells, which were