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Comparison of two digestion strategies on characteristics and differentiation potential of human dental pulp stem cells

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ABSTRACT

Objective: : This study aimed to compare the behavior of dental pulp stem cells (DPSCs) after isolation using solutions containing either collagenase/dispase or collagenase alone.
Design: : DPSCs were isolated by two digestion methods (collagenase/dispase or collagenase alone) from human third molars. Immunophenotypic features were confirmed by flow cytometry for cell markers STRO-1, cluster of differentiation (CD) 146, CD45, and collagen type-I. The proliferation potential of cells was evaluated by 5-bromo-2′-deoxyuridine (brdU) incorporation assay, and finally they were assessed for multi-lineage differentiation potential. Data were analyzed using one-way analysis of variance and independent *t*-tests.
Results: : DPSCs isolated by either method showed similar levels of STRO-1, CD45, and collagen type-I and similar incorporation of brdU ($P > 0.05$). However, DPSCs obtained by collagenase I/dispase treatment had significantly higher numbers of $CD146^+$ cells and osteogenic and chondrogenic capacities compared to those obtained by treatment with collagenase I alone ($P < 0.05$). On the other hand, more STRO-1 + /CD164-DPSCs were found in the collagenase alone group with higher adipogenic potential.
Conclusions: : Different enzyme solutions gave rise to different populations of DPSCs. Dispase enhanced isolation of $CD146^+$ DPSCs probably by disrupting the basement membranes of blood vessels and releasing DPCSs embedded in the perivascular niche. Furthermore, the differentiation potential of DPSCs was influenced by the change in enzyme solution.

1. Introduction

Since the primary isolation of stem cells from human pulp tissue in 2000 (Gronthos, Mankani, Brahim, Robey, & Shi, 2000), many different other populations of dental mesenchymal stem cell like cells have been extracted from different parts of post-natal human teeth (Huang, Gronthos, & Shi, 2009) Although the therapeutic applications of bone marrow and adipose stem cells have been extensively studied, the discovery of stem cells in the tooth have led to renewed hope in regenerative medicine (Casagrande, Cordeiro, Nör, & Nör, 2011; da Silva Meirelles, Chagastelles, & Nardi, 2006; Dominici et al., 2006). Among these tooth-related stem cells, DPSCs have been widely studied for their potential in pulp and dentin regeneration (Gronthos et al., 2002; Huang & Garcia-Godoy, 2014). They have been recognized as infrequent populations of highly proliferative stem cells located within larger populations of more committed progenitors.

Pulp cells can be isolated by either outgrowth technique or enzymatic digestion technique (Huang, Sonoyama, Chen, & Park, 2006; Yildirim, 2013). Although the outgrowth method is a more convenient technique than enzyme digestion, the latter method is the method of choice for isolation of mesenchymal stem cells derived from of dental tissue (Bakopoulou et al., 2011; Ferrúa et al., 2017). Furthermore, Both DPSCs and deciduous teeth stem cells isolated with the enzyme digestion method had higher proliferation, differentiation and mineralization capacities than those isolated by the outgrowth method (Huang et al., 2006; Karamzadeh, Eslaminejad, & Aflatoonian, 2012).

Collagenase type I and dispase are the most commonly used enzymes in the enzyme digestion method. In this combination, collagenase I mostly breaks down collagen type I, and dispase mainly cleaves fibronectin and collagen IV (Fogarty & Griffin, 1973; Kühn, 1995). Since the first time dental pulp stem cells were isolated by Gronthos et al. (2002) using a combination of collagenase type I and dispase, this enzyme solution has been the one most commonly used for the isolation of dental mesenchymal stem cells. According to a recent

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systematic review regarding DPSC isolation, collagenase type I and dispase were the enzymes of choice in 54.4% of studies, while other enzyme types were selected in about 45% of studies. When collagenase type I and dispase were not chosen, over 17% of researchers preferred to use collagenase I as the only enzymatic agent (Ferrúa et al., 2017).

Using collagenase type I alone has not been limited to the isolation of DPSCs. For example, other dental mesenchymal stem cells such as Human deciduous dental pulp cells (Govindasamy et al., 2010; Mikami et al., 2011; Nourbakhsh et al., 2011), Human stem cells from apical papilla (Yan et al., 2014), Human periodontal ligament stem cells (Núñez-Toldrà et al., 2017) also have been isolated successfully with collagenase type I.

In 1991, Nakashima compared four different enzyme solutions (trypsin, trypsin and collagenase, collagenase, and collagenase then trypsin) in the isolation of dental pulp cells. He showed that cell viability and growth of cells is significantly affected by the type and combination of enzymes (Nakashima, 1991). However, other characteristics of these cells were not evaluated in this study. Since then, dental-derived mesenchymal stem cells have been discovered, and new insight into the characteristics and behavior of these cells has been obtained. Isolation techniques and enzymes have also been changed. To the extent of our knowledge, since Nakashima's study, no comparison has been made between characteristic of cells isolated with different currently used enzyme solutions. For the isolation of DPSCs, twenty different enzyme solutions have been developed and used successfully (Ferrúa et al., 2017). This diversity can yield a variety of isolated cells, resulting in misinterpretations of their characteristics, behaviors, and biological processes, as can the comparison of the results of different studies. Therefore, the aim of this study was to compare growth and the immunophenotypic and differentiation characteristics of DPSCs extracted with collagenase/dispase and collagenase alone, the two most common enzyme solutions used for isolating DPSCs.

2. Materials and methods

2.1. Primary cell culture

Intact human impacted third molars were collected from healthy patients (10 donors aged 15–20 yrs) in the Dental Clinics at the Isfahan Azad University under the approved guidelines and protocol. All experiments were conducted after patients had signed informed consent forms. A high-speed bur was used to make a notch around each tooth at the level of the cementoenamel junction without invading the pulp tissue. The crown was separated using an elevator and the pulp was removed and immersed in phosphate buffered saline. Before establishment of culture, tissues were separately washed three times in phosphate buffered saline and minced. To compare two enzymatic digestion methods, pulps were equally divided into part A, which was treated with collagenase type I (4 mg ml^{-1}) for 60 min at 37 °C, and part B, which was treated with a combination of collagenase type I (3 mg ml^{-1}) and dispase (4 mg ml^{-1}) for 60 min at 37 °C (all from Gibco, Paisley, UK). Single-cell suspensions were prepared by passing the cells through a 70-μm strainer and grown in α-Modification of Eagle's Medium (α-MEM; Sigma, Munich, Germany) supplemented with 15% fetal bovine serum, 1% L-glutamine, 100 U ml^{-1} penicillin, and 100 mg ml^{-1} streptomycin (all from Gibco, Paisley, UK) at 37 °C, 5% CO2, and were allowed to reach 85% confluency. Subsequently, the cells were subcultured in Dulbecco's Modified Eagle's medium (DMEM; Gibco, Paisley, UK) high glucose (4.5 g/L) using the same supplement, substituting 15% fetal bovine serum with 10% fetal bovine serum, at a ratio of 1:3 continuously. All experiments were carried out by using DPSCs from the independent donors at passages 3–6.

2.2. Immunophenotype characterization

The immunophenotypic features of isolated cells were confirmed by flow cytometry as described earlier (Nourbakhsh et al., 2011). Briefly, cells were dissociated with TrypLE, and cell suspensions were stained by various antibodies against mesenchymal stem cell markers, including STRO-1, CD146, CD45, and collagen type-I. Cells were labeled with isotype-matched antibodies and then served as background controls. Subsequently, cells were treated with suitable secondary antibody, and samples were submitted to the FACSCalibur cytometer (Becton Dickinson, San Jose, CA, USA) by collecting 10,000 events per sample at a flow rate of around 100 events per second. The data analysis was performed using WinMDI 2.9 software.

2.3. Multilineage differentiation

To achieve osteogenic differentiation, cells were initially cultured at a density of 2×10^4 cells/cm^2 in Dulbecco's Modified Eagle's medium. Quiescent cultures of confluent cells were then incubated for three weeks in the presence of 10 nM dexamethasone, 50 mg ml^{-1}ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate (all from Sigma, Munich, Germany). At the end of the differentiation period, calciumaccumulation was detected via Alizarin Red staining. For Alizarin Red staining, the cells were washed twice with Dulbecco's phosphate buffered saline and fixed in methanol (Merck, Darmstadt, Germany) for 10 min at room temperature. Cells were then stained with Alizarin Red S (Merck, Darmstadt, Germany) 2% (pH 4.1–4.3) for 20 min and washed two times with dried delactosed whey for 5 min.

Adipogenesis was induced in cultures by addition of differentiating medium to confluent cells containing basal α-Modification of Eagle's medium plus 100 nM dexamethasone, 50 μg ml^{-1} indomethacin, and 50 μg ml^{-1}ascorbic acid 3-phosphate (both from Sigma) for 21 days with medium changes three times per week. Oil droplets that accumulated within cells after adipogenic differentiation were stained by Oil Red O reagent. For Oil Red staining, after removing the medium and washing the cells with Dulbecco's phosphate buffered saline, cells were immersed in the 4% paraformaldehyde for 30 min and then, gently washed with dried delactosed whey. The fixation procedure was completed by adding 60% isopropanol (Merck, Darmstadt, Germany) for 5 min. The lipid droplets were detected by staining the cells in 0.2% Oil Red-O (Merck, Darmstadt, Germany) for 15 min, followed by carefully washing with dried delactosed whey. All the procedures were performed at room temperature.

For chondrogenic differentiation, cells were cultured as a micromass pellet of cells in the presence of chondrogenic differentiation medium (Lonza, Belgium) with transforming growth factor beta-3 for three weeks. At the end of the cultivation period, cartilaginous extracellular matrix was evaluated using toluidine blue (1% toluidine blue/1% sodium borate, Merck, Darmstadt, Germany) staining of pellet cryosections (6 μm thickness) for 5 min at room temperature.

Quantitation of total red image intensity was determined using ImageJ software from 3 fields per each sample and shown as a percentage of Alizarin Red- and Oil Red-stained area over total area.

2.4. BrdU incorporation assay

To evaluate cell proliferation in DPSCs, cultures were incubated with 10 μM brdU (bdrU, Sigma, Munich, Germany) overnight at 37 °C. After the incubation phase, the labeling solution was removed, cells were harvested and fixed, and DNA was denatured. This process was followed by the detection level of cell-associated brdU by the addition of mouse anti-bdrU antibody (Sigma, Munich, Germany) to determine the level of DNA synthesis. The flow cytometry analysis of the cells was carried out using the FACSCalibur cytometer (Becton Dickinson, San Jose, CA, USA) and analyzed by WinMDI 2.9 software. BrdU-labeled cells were also viewed and analyzed by fluorescence microscopy (Olympus, BX51, Japan).