Isolation and Characterization of Human Dental Pulp Stem Cells from Cryopreserved Pulp Tissues Obtained from Teeth with Irreversible Pulpitis

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Abstract

Introduction: Human dental pulp stem cells (DPSCs) are becoming an attractive target for therapeutic purposes because of their neural crest origin and propensity. Although DPSCs can be successfully cryopreserved, there are hardly any reports on cryopreservation of dental pulp tissues obtained from teeth diagnosed with symptomatic irreversible pulpitis during endodontic treatment and isolation and characterization of DPSCs from such cryopreserved pulp. The aim of this study was to cryopreserve the said pulp tissues to propagate and characterize isolated DPSCs. **Methods:** A medium consisting of 90% fetal bovine serum and 10% dimethyl sulfoxide was used for cryopreservation of pulp tissues. DPSCs were isolated from fresh and cryopreserved pulp tissues using an enzymatic method. Cell viability and proliferation were determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. DPSC migration and interaction were analyzed with the wound healing assay. Mesenchymal characteristics of DPSCs were verified by flow cytometric analysis of cell surface CD markers. The osteogenic and adipogenic potential of DPSCs was shown by von Kossa and oil red O staining methods, respectively, and the polymerase chain reaction method. **Result:** We found no significant difference in CD marker expression and osteogenic and adipogenic differentiation potential of DPSCs obtained from fresh and cryopreserved dental pulp tissue. **Conclusions:** Our study shows that dental pulp can be successfully cryopreserved without losing normal characteristics and differentiation potential of their DPSCs, thus making them suitable for dental banking and future therapeutic purposes. *(J Endod 2016;42:76–81)*

Key Words

Cryopreservation, dental pulp, dental pulp stem cells, endodontics, mesenchymal stem cells, stem cell banking

Dental pulp stem/progenitor cells originating from neural crests exist in the pulp tissue of teeth (1). In 2000, Dr Gronthos and colleagues identified stem cells in human dental tissues for the first time and reported isolation of postnatal human dental pulp stem cells (DPSCs) by enzymatic treatment (2). These DPSCs were found to be capable of differentiating into neural-like cells *in vitro* and were also capable of forming ectopic dentin and associated pulp tissue *in vivo* (3). Subsequent studies revealed that these newly identified cells are a clonogenic and rapidly proliferative population of mesenchymal stem cells (MSCs). By definition, MSCs have the ability to differentiate into osteoblasts, adipocytes, and chondrocytes; adhere to plastic tissue culture dishes; and express CD105, CD73, and CD90 but not CD45, CD34, CD14 or CD11b, CD79a or CD19, or HLA-DR surface molecules (4). All of these vital features of MSCs have been observed in DPSCs and confirmed through several characterization studies (5, 6).

Dental pulp tissue is an attractive source of MSCs because it is a noncontroversial and readily accessible source. Because regeneration and maintenance are reliant on MSCs (7), these features of DPSCs bring to light their promise in the field of tissue engineering and regenerative medicine, in particular regenerative endodontics (revascularization/pulpal regeneration) (8).

It is known that DPSCs possess immunomodulatory effects (9) and can be obtained from inflamed dental pulps as well. Alongi et al (10) showed that DPSCs isolated from inflamed pulps can express higher levels of MSC markers CD90 and CD105 in comparison with those from normal pulps. Because successful isolation of DPSCs has been limited to 5 days after tooth extraction, it is important to optimize their isolation and preservation (11).

It is well documented that cryopreserved whole teeth can be used as a possible source of DPSCs (12, 13). In 1 of these related studies, scientists cryopreserved whole teeth after excavating microchannels into the tooth with laser piercing to allow the cryopreservative access to the dental pulp and preserve the cells. Their data showed that isolated DPSCs from these cryopreserved teeth exhibit MSC morphology, immunophenotype, viability, and a proliferation rate comparable with those of cells isolated from fresh, noncryopreserved teeth (13). In another study, instead of whole teeth cryopreservation, it has been shown that cryopreserved dental pulp tissues of deciduous teeth are also a feasible stem cell resource for isolation of dental stem cells from human exfoliated deciduous teeth (14).

To develop DPSC banking, the primary step is to identify and select the best accessible sources of pulp tissue for isolation of DPSCs. Although isolation of DPSCs from teeth diagnosed with irreversible pulpitis is almost impractical immediately after endodontic therapy, it has been proven that the inflammatory process does not affect the

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properties of the DPSCs (15). Therefore, cryopreservation of pulp tissues from teeth with irreversible pulpitis and subsequent isolation of DPSCs from them would be an alternative with therapeutic value to overcome this issue.

Hence, in the present study, we aimed to evaluate whether cryopreserved inflamed pulp tissues of permanent teeth with irreversible pulpitis are a retrievable and practical DPSC source? With this aim, we attempted to compare characterization of DPSCs isolated from cryopreserved and noncryopreserved (fresh) pulp tissues of permanent teeth with symptomatic irreversible pulpitis.

Materials and Methods

Pulp tissues were obtained during root canal treatment of human permanent carious molar teeth of 15- to 30-year-old patients with symptomatic irreversible pulpitis at the Department of Conservative Dentistry and Endodontics, Sri Rajiv Gandhi College of Dental Sciences and Hospital, RGC Campus, Bangalore, India. Informed consent was obtained from donors, and the protocol was approved by the Institutional Ethical Committee of Sri Rajiv Gandhi College of Dental Sciences and Hospital.

Isolation of DPSCs

DPSCs were isolated from cryopreserved (n = 10) and noncryopreserved (fresh) human dental pulp tissues (n = 10). Dental pulp tissues were washed 2 to 3 times with Dulbecco phosphate-buffered saline solution under the laminar flow chamber. Then, 2-mg/mL collagenase blends (Sigma-Aldrich, St Louis, MO) were added, and the tissues were minced to smaller pieces to increase the surface area for better enzyme action. Then, minced tissues were kept in the incubator (5% CO_2, 37°C) for 1 hour. After incubation, the action of enzymes was neutralized by addition of the culture medium. The samples were then centrifuged at 400g for 5 minutes, and cell pellets were collected and plated in 24-well plates and kept in the incubator. Cultures were fed with fresh media every 48 hours.

DPSCs were cultured in knockout Dulbecco modified Eagle medium (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 5 mmol/L L-glutamine (Gibco), and 50 U/mL penicillin streptomycin (Gibco).

Cryopreservation of Dental Pulp Tissues

Dental pulp tissues were mixed with chilled cryopreserved medium (4°C) and kept overnight at −80°C. The cryopreserved medium consisted of 10% dimethyl sulfoxide (Sigma-Aldrich) and 90% fetal bovine serum (Hyclone). They were transferred into liquid nitrogen in a controlled cooling manner enabling a cooling rate of 1°C/min and stored. After 3 months of storage, the cryopreserved pulps were thawed out, and DPSCs were isolated using the previously mentioned method.

Growth Kinetic Study

The growth kinetic study was performed by calculating population doubling (PD), cumulative population doubling (CPD), and population doubling time (TD). DPSCs were dissociated with 0.25% trypsin (Gibco) and counted using the trypan blue exclusion method on a Neubauer hemocytometer at the end of each passage once cells were about 90% confluent, and then they were replated for the subsequent passages.

The PD and TD were determined by the following formulas, respectively: PD = LogNh−LogNi/Log2 and TD = t*Log_2/LogNh−LogNi, where Ni = number of cells at seeding time, Nh = number of cells at harvesting time, and t = culture time (in hours), and CPD = PD of each passage + PD of previous passage.

MTT Assay

It was a 2-day protocol. On the first day, 7500 cells mixed with 100 μL growth media were seeded into each well of a 96-well plate and incubated overnight. The next day 20 μL of 5 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was added to each well, and cells were incubated for 3.5 hours at 37°C in the culture hood. Then, media was removed, and 150 μL MTT solvent was added. The plate was covered with tinfoil, and cells were agitated on an orbital shaker for 15 minutes. Absorbance was read at 590 nm with a reference filter of 620 nm.

Wound Healing Assay

The effects of cryopreservation of pulp on the migration of stem cells were examined via the wound healing assay. For this purpose, DPSCs were mechanically scratched with a sterile pipette tip in 6-well plastic dishes to remove a certain portion of cells. The migratory ability of the cells was assessed by counting the number of cells within the scratched wound at 0, 12, 24, 36, and 48 hours.

Flow Cytometric Analysis

Flow cytometry with DPSCs was performed to characterize these cells for a candidate set of MSC-specific surface markers. Cells were harvested and counted; then, 10 μL phycoerythrin/fluorescein isothiocyanate (PE/FITC) tagged antibodies were added into the appropriate number of cells. Samples were stained for 1 hour at 4°C and then washed with fluorescence-activated cell sorting (FACS) buffer for 5 minutes. To fix the cells, 4% paraformaldehyde was added to the pellet and resuspended. The following markers were used for immunostaining: CD166-PE, CD 105 FITC, CD73-PE, CD90-PE, CD34-FITC, CD19-PE, CD45-PE, and HLA-DR-PE (Table 1). Data analysis was optimized against control cells incubated with specific isotypes immunoglobulin (Ig) G1-PE, IgG2a-PE, and IgG1-FITC on FACS Calibur (BD Biosciences, Franklin Lakes, NJ). Cells were identified by light scatter for 10,000 gated events and analyzed using BD Cell Quest Pro software (BD Biosciences).

TABLE 1. The List of Conjugated Antibodies Used in the Study

Antigen	Antibody	Dilution	Brand	Application
CD166	PE conjugated antihuman CD16	1:20	BD Biosciences	Immunophenotyping by flow cytometry
CD105	FITC conjugated antihuman CD105	1:20	BD Biosciences	Immunophenotyping by flow cytometry
CD90	PE conjugated antihuman CD90	1:20	BD Biosciences	Immunophenotyping by flow cytometry
CD73	PE conjugated antihuman CD73	1:20	BD Biosciences	Immunophenotyping by flow cytometry
CD45	PE conjugated antihuman CD45	1:20	BD Biosciences	Immunophenotyping by flow cytometry
CD34	FITC conjugated antihuman CD34	1:20	BD Biosciences	Immunophenotyping by flow cytometry
CD19	PE conjugated antihuman CD19	1:20	BD Biosciences	Immunophenotyping by flow cytometry
HLA DR	PE conjugated antihuman HLA-DR	1:20	BD Biosciences	Immunophenotyping by flow cytometry

FITC, fluorescein isothiocyanate; HLA-DR, human leukocyte antigen-DR; PE, phycoerythrin.