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Simplified conditions for storing and cryopreservation of dental pulp stem cells

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ABSTRACT

Objectives: This study aimed to simplify the collection, isolation and cryopreservation procedure of human dental pulp stem cells (DPSCs) to ease the establishment of dental stem cell banking.
Design: Extracted third molars were collected and stored either in growth medium or in gentamicin-saline (480 μg/ml) for 6, 9 or 12 h. DPSCs were isolated and subjected to cryopreservation by a controlled-rate or rapid freezing method in 5 or 10% DMSO. Flow cytometry and growth pattern of DPSCs before and after cryopreservation were conducted.
Results: Rate of contamination by which the extracted teeth were stored in control and gentamicin-saline were 9.1% (N = 33) and 2.3% (N = 43), respectively. Successful cell isolation rate of teeth preserved in gentamicin-saline at 6 h (92.9%) was comparable to those of growth media group (90.3%). At 9 and 12 h, the rates dropped significantly to 75% and 54%, respectively. Cryopreservation by controlled-rate freezing either in 5 or 10% DMSO resulted in a significantly higher percentage of viable cells than by rapid freezing. Cells conserved by controlled-rate freezing in 5% DMSO showed a pattern of growth similar to control unfrozen cells; 10% DMSO significantly deteriorated the growth pattern of the cells. After thawing, DPSCs conserved by controlled-rate freezing still expressed stemness characteristics, although hematopoietic stem cell markers were slightly increased.
Conclusion: Gentamicin-saline was effective in preserving human teeth for DPSC isolation. Controlled-rate freezing in 5% DMSO gave the highest rate of cell viability. This study simplifies the storage conditions and proposes a simple method for cryopreservation of DPSCs.

1. Introduction

Dental derived stem cells, especially dental pulp stem cells (DPSCs), are an accessible source of autologous adult stem cells that can be isolated from extracted wisdom teeth and teeth removed for orthodontic reasons. To ensure stem cell viability and characteristics, the collection and isolation processes are quite critical and cannot be easily performed routinely in an ordinary dental clinic.

DPSCs were first isolated and characterized by Gronthos et al. in 2000. Recently human DPSCs were shown to be multipotent and express multilineage differentiation potential including osteo/odontogenic, adipogenic, neurogenic, chondrogenic, myogenic lineages. Moreover, it was possible to differentiate these cells into induced pluripotent stem (iPS) cells (d'Aquino et al., 2007; Fitzgerald, Chiego, & Heys, 1990; Gronthos et al., 2002; Gronthos, Mankani, Brahim, Robey, & Shi, 2000; Tamaoki et al., 2010). Despite being an ideal source of cells for tissue engineering, DPSCs are often overlooked due to the complex collection procedure from the dental clinic to the laboratory (Kadar et al., 2009). Several studies attempted to optimize the collection and cryopreservation procedure of DPSCs (Gioventu et al., 2012; Huang, Yang, Wang, & Lee, 2010; Perry et al., 2008; Thirumala, Goebel, & Woods, 2009). The previously reported standard methods required growth media for tooth storage and relatively high doses of DMSO for cryopreservation. Such storage media and cryopreserving substances are not readily available in dental clinics and, therefore, hamper DPSC isolation and utilization.

Previous studies reported bacterial contamination of dental tissues following the extraction and their transportation in the storage media to the laboratories (Poi et al., 2013). Here, we suggest the use of gentamicin to reduce contamination. Gentamicin belongs to the

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aminoglycosides class of antibiotics which are active against a wide range of bacterial infections (Kadar et al., 2009; Zhang, Walboomers, Shi, Fan, & Jansen, 2006). Following an initial culture of DPSC, liquid nitrogen and cryoprotectants (CPA) have been used to keep cells viable in a deep freezing condition for a long period of time (Oh et al., 2005; Perry et al., 2008). Dimethyl sulfoxide (DMSO) is the most widely used cryoprotectant for many cell types including stem cells obtained from umbilical cord blood, dental pulp and placenta. The optimal concentration of DMSO for cryopreservation has been reported to be 10% v/v (Huang et al., 2010). Still, the toxicity of DMSO is of serious concern when the cells are thawed prior to their clinical usage. DMSO has also been reported to affect differentiation of stem cells (Thirumala, Goebel, & Woods, 2013). Therefore, the optimum dose of DMSO that minimally affect the viability of cryopreserved cells should be carefully calibrated.

In the present study, we intended to optimize the process of DPSC collection, isolation, characterization, and cryopreservation in a practical way from the dental clinic to the laboratory. We tested the effect of a simplified storage solution (gentamicin in sterile saline) on preserving the tooth for DPSC isolation at 6, 9, and 12 h after extraction. The cells were then frozen in 5 or 10% DMSO either by controlled-rate or rapid freezing and kept in liquid nitrogen for 6 months. The characteristics of the DPSCs prior to and after freezing were analyzed by trypan blue staining, measuring growth rate and examination of stem cell markers. This study will propose optimized methods to ease the utilization of DPSCs and the establishment of stem cell banking.

2. Materials and methods

2.1. Isolation and culture of primary human dental pulp stem cells

Human primary DPSCs were cultured and characterized as previously described (Ha et al., 2011; Tran Hle and Doan, 2015). Third molars from healthy, 18 to 25-year-old individuals, were extracted as recommended by their dentists. Each subject was without systemic and oral infection or diseases and the molars had no caries. Immediately after extraction, each tooth was transferred to the lab either in ice cold control medium (DMEM/F12 with 10% FBS, 1% L-Gluamine, 300 U/ml penicillin and 300 mg/ml streptomycin, Sigma, St. Louis, MO) or gentamycin-saline solution (480 µg/ml gentamicin sulfate in NaCl 0.9%). The extracted teeth were kept in storage solutions for 6, 9 and 12 h before further processing for DPSC isolation (Fig. 1). To avoid contamination, the soft tissue attached to the cervical area was carefully removed. The extracted teeth were rinsed twice in Dulbecco's phosphate-buffered saline without calcium and magnesium (PBS) and cut with a handpiece to obtain the pulp tissue. The dental pulp tissues were minced into 2 × 2 mm^2 fragments. The tissue explants were placed in 35 mm flasks with culture medium (DMEM/F12, 10% FBS, 1% L-Gluamine, 1% antibiotics) and cultured until outgrowing cells reached confluence. The cultures were kept at 37 °C in a humidified atmosphere with 5% CO_2, the medium was replaced every 3 days. The primary human DPSCs at the 4th passage were used for the following experiments.

The patients provided written consent for the use of discarded tissues for research purposes. Tissue samples were de-identified and analyzed anonymously. The Ethics Committee of the University of Medicine and Pharmacy at Ho Chi Minh City, Vietnam approved the study to be carried out according to the protocol and informed permission dated and/or amended as follows in compliance with the ICH/GCP (76/DHYD-HD 09/08/2013).

2.2. Flow cytometry

The 4th passage of cells were harvested with trypsin-EDTA 0,25% and resuspended in washing buffer. For cell surface staining, 1 ml of 10^6 cell suspension was incubated with 10 µl FITC-conjugated anti-CD13, CD14, CD34, CD44, CD45, CD105 and HLA-DR antibodies; PerCP-CyTM5.5-conjugated anti-CD90 antibody and PE-conjugated anti-CD73 antibody (BD Biosciences Pharmingen, San Diego, CA). A non-immune isotype antibody was used as negative controls. After staining, the cells were washed in wash buffer and then fixed with 1% paraformaldehyde. Data analysis was performed using FACS Calibur by laser argon 250 MW at 488 nm and CellQuest software (BD Bioscience).

2.3. Viable cell counting and measuring the growth curve

Twenty microliter of cell suspension was mixed 1:1 with 0.4% trypan blue solution in PBS and incubated for 3 min at room temperature, after which they were loaded into a clean, and dry hemocytometer and observed by a microscope at 40× magnification. Non-viable cells were stained dark blue. Cell viability was calculated as the number of unstained or viable cells divided by the total number of cells

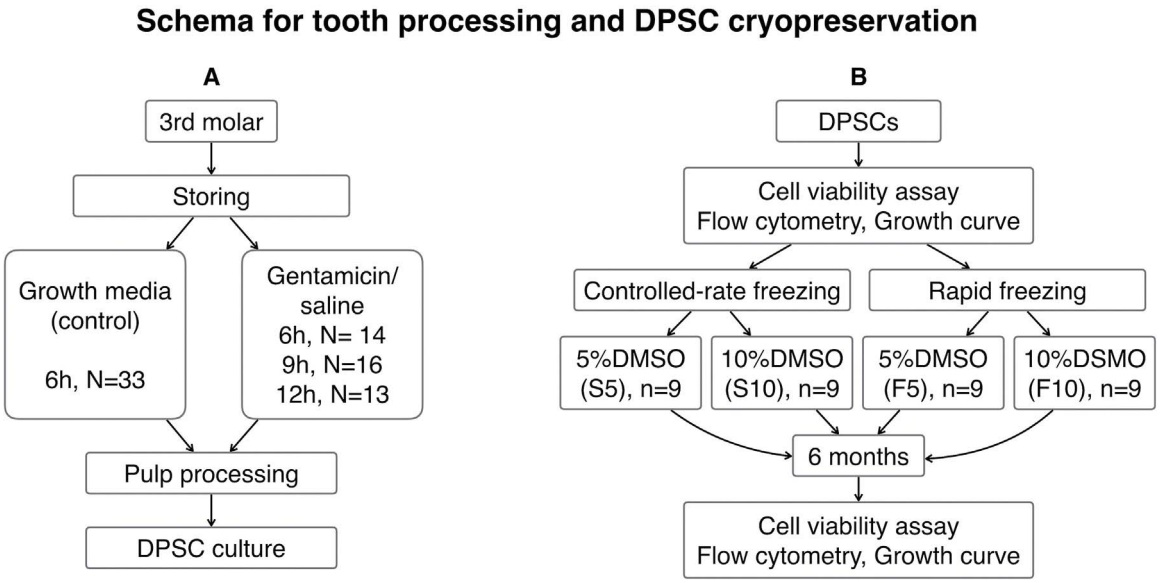

Fig 1. Scheme of the experiments: tooth processing and DPSC culture from teeth stored in growth medium and gentamicin solution (A). DPSC cryopreservation by controlled-rate freezing and rapid freezing methods in 5% or 10% DMSO; and evaluation process of cryopreserved cells by cell viability assay, flow cytometry and growth rate (B).