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Cell viability and proliferation capability of long-term human dental pulp stem cell cultures

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Abstract

Background aims. Evaluation of cell viability is one of the most important steps of the quality control process for therapeutic use of cells. The aim of this study was to evaluate the long-term cell viability profile of human dental pulp stem cell (hDPSC) subcultures (beyond 10 passages) to determine which of these passages are suitable for clinical use and to identify the cell death processes that may occur in the last passages. *Methods.* Four different cell viability assays were combined to determine the average cell viability levels at each cell passage: trypan blue exclusion test, water-soluble tetrazolium 1 (WST-1), LIVE/DEAD Viability/Cytotoxicity Kit and electron probe x-ray microanalysis (EPXMA). Apoptosis was assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and caspase 4 and BCL7C Western blotting, and cell proliferation was analyzed by WST-1 and proliferating cell nuclear antigen protein detection. *Results.* hDPSCs showed high average cell viability levels from passages 11–14, with adequate cytoplasmic and mitochondrial functionality at these subcultures. A non-significant trend to decreased cell proliferation was found from passages 16–20. EPXMA and TUNEL analyses suggested that a pre-apoptotic process could be activated from passages 15–20 ($P < 0.001$), with a correlation with caspase 4 and BCL7C expression. *Conclusions.* hDPSCs corresponding to passages 11–14 show adequate cell function, proliferation and viability. These cells could be considered as potentially useful for clinical applications.

Key Words: *cell viability, dental pulp, electron-probe x-ray microanalysis, mesenchymal stromal cells*

Introduction

Human dental pulp stem cells (hDPSCs) have been known only since 2000, and these cells have been studied in depth and characterized since then (1). hDPSCs are considered to be the most feasible and promising stem cells derived from human teeth because of their biologic properties, because they are easy to obtain, and because they are present during almost the whole life span in the dental pulp of permanent human teeth. hDPSCs are clonogenic and highly proliferative, even more than bone marrow mesenchymal stromal cells (2). hDPSCs are very similar to other adult mesenchymal stromal cells in regard to the positive expression of mesenchymal undifferentiation markers and the negative expression of CD45 (3,4). hDPSCs have high differentiation potential; they have been shown to have great capabilities for differentiating to chondrogenic, osteogenic, adipogenic, neurogenic and epithelial lineages (1,5–8). Because of this differentiation potential, these cells are globally considered as mesenchymal stromal cells (2,4,6,9–12). Similar to other stem cells, hDPSCs are increasingly used in regenerative medicine, especially for the development of biologic substitutes (artificial tissues) by tissue engineering (5,8,13–19).

It is necessary to ensure the proliferative and regenerative potential of cells used in regenerative medicine and tissue engineering protocols to guarantee the success of these protocols (20–24). Evaluation of cell viability is one of the most important goals of the quality control process for cells used for the generation of artificial tissues. Understanding the specific cell death mechanisms that may occur during sequential cell culturing would contribute to a better selection of the most appropriate cell sources.

Several methods have been used to evaluate cell viability, ranging from commonly used methods based on the analysis of the integrity of the cell membrane

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employing vital exclusion dyes such as trypan blue (25,26) to other assays focused on the study of cell metabolism such as calcein acetoxymethyl ester (AM) staining or water-soluble tetrazolium 1 (WST-1) methods (27). Electron probe x-ray microanalysis (EPXMA) allows the accurate determination of cell viability and the identification of the mechanisms underlying cell death by quantification of the ionic elements that play a key role in cell viability. This method has been extensively used for the study of cell viability of different cell types, including hDPSCs (28–30).

Adult stem cells cultured in the laboratory for several passages tend to show a typical three-step cell viability profile as previously reported (20,22,23): (i) an initial adaptation to *ex vivo* cell culture conditions associated with a slight decrease of cell viability, (ii) an increasing period when cell viability increases and cells reach the top cell viability levels (cells during this period are recommended for use in regenerative medicine) and (iii) a decreasing phase when cells tend to lose viability and cell death is present (it is not recommended to use cells during this period).

hDPSC viability was studied more recently during the first 10 cell subcultures (23) by using an array of cell viability assays. However, hDPSCs reached the top cell viability at the end of the study (passage 9). It is necessary to go further in this type of research and to analyze long-term hDPSC subcultures (beyond 10 passages) to determine if these passages are also suitable for clinical use and because it is important to identify subcultures in which cells undergo active cell death and the mechanisms underlying this process. Once the cell viability profile has been evaluated, researchers will be able to choose hDPSC subcultures with optimal cell viability and proliferation and discard low-viability subcultures for therapeutic use.

The aim of this study was to evaluate the cell viability profile of long-term hDPSC subcultures (from passages 11–20) to determine the putative usefulness of these cells and to identify the stage when hDPSCs tend to lose cell viability. In addition, the mechanisms involved in cell death and its early indicators were analyzed.

Methods

Samples and hDPSC cultures

Four human dental pulps were obtained from young adult teeth (age range, 18–30 years). All teeth were third molars without dental or periodontal pathology extracted by dental prescription at the School of Dentistry of the University of Granada. All teeth demonstrated a fully developed root stage (closed apex stage).

After extraction, teeth were stored at 4°C in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) supplemented with 600 U/mL penicillin, 0.6 mg/mL streptomycin and 1.5 μg/mL amphotericin B (Sigma-Aldrich Chemie GmbH) and immediately processed at the Tissue Engineering Laboratory at the School of Medicine of the University of Granada. Dental pulps were extracted and digested as previously described (2). The medium used for hDPSC culture was DMEM supplemented with 10% fetal bovine serum (catalog number F9665, lot number 062M3398; Sigma-Aldrich, St Louis, MO, USA) and 1% antibiotic and antimycotic solution (Sigma-Aldrich). Medium was changed every 3 days, and cells were subcultured at sub-confluence using a 0.5 g/L trypsin and 0.2 g/L ethylenediamine tetraacetic acid solution (Sigma-Aldrich) at 37°C for 4 min. Cells were kept in culture until passage 20 and analyzed from passages 11–20.

Trypan blue vital dye exclusion assay

To determine the number of cells and their viability using trypan blue, 20 μL of trypsinized and re-suspended cells were mixed with 20 μL of 0.4% solution of trypan blue dye (Sigma-Aldrich) for 1 min. Cells were immediately counted using a Neubauer microchamber (Brand GmbH, Wertheim, Germany) with a light microscope. All counts were done using four technical duplicates of each sample. Means and standard deviations were calculated for each subculture.

WST-1 metabolic proliferation and cell viability assay

To determine the mitochondrial metabolic activity and cell proliferation of hDPSCs, the WST-1 method was used as previously described (22,23). Briefly, 10,000 cells were cultured in 96-well plates (Iwaki Laboratories, Iwaki, Japan). After subsequent subculture for 48 h, cells were rinsed with phosphate-buffered saline (PBS), and 10% WST-1 reagent solution (Roche Diagnostics, Indianapolis, IN, USA) was added for 4 h at 37°C. Each sample was analyzed using a UVM 340 Microplate Reader (Asys, Cambridge, UK). As negative controls, selected cultured wells were previously treated with 2% Triton X-100 (Probus, Barcelona, Spain). All counts were done using four technical duplicates of each sample.

LIVE/DEAD cell viability assay

Simultaneous evaluation of cytoplasmic function and membrane integrity was carried out using the LIVE/DEAD Viability/Cytotoxicity Kit for mammalian

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