EVALUATION OF TWO HUMAN DENTAL PULP STEM CELL CRYOPRESERVATION METHODS

Juan C. Munévar, Nicole Gutiérrez, Nury T. Jiménez, Gloria I. Lafaurie

Unit of Basic Oral Investigation-UIBO. Faculty of Dentistry. El Bosque University. Bogota, Colombia.

ABSTRACT

Dental pulp is a promising source of mesenchymal stem cells for use in cell therapy and regenerative medicine. Methods for storing stem cells with minimum compromise of cell viability, differentiation capacity and function should be developed for clinical and research applications. The aim of this study was to evaluate whether human dental pulp stem cells (hDPSCs) isolated and cryopreserved for 1, 7 and 30 days maintain viability and expression of specific stem cell markers. Human dental pulp stem cells were isolated from 23 healthy patients aged 18 to 31 years. Dental pulp was enzymatically dissociated, and CD105+ cells were separated using the Miltenyi™ system. The hDPSCs were cryopreserved using the Kamath and Papaccio methods. Post-cryopreservation viability was measured by flow cytometry (7AAD) and by the expression of the phenotype markers CD105+/ CD73+, CD34-/CD45-. The Papaccio method showed greater cell viability for cells that had been frozen for 30 days (59.5%) than the Kamath method (56.2%), while the Kamath method provided better results for 1 day (65.5%) and 7 days (56%). Post-cryopreservation expression of the markers CD105+/CD34- was greater after 1 and 7 days with the Kamath method and CD105+/CD45- were expressed after all 3 cryopreservation times. There was greater expression of CD73+ in the hDPSCs after 1 and 7 days with the Kamath method, and after 30 days with the Papaccio method.

These results suggest that hDPSCs express mesenchymal stem cell markers after cryopreservation. However, cryopreservation time may affect marker expression, probably by altering the spatialconfiguration of cell membrane proteins or by compromising cells at a certain level of differentiation.

Key words: *Cryopreservation, Mesenchymal stem cells, Phenotype, Cell viability, Dental pulp, Regenerative medicine.*

EVALUACIÓN DE DOS MÉTODOS DE CRIOPRESERVACIÓN DE CÉLULAS TRONCALES DE PULPA DENTAL HUMANA

RESUMEN

La pulpa dental es una fuente promisoria de células madre mesenquimales para ser utilizadas en terapia celular y medicina regenerativa. El desarrollo de métodos que permitan almacenar las células madre con mínimo compromiso de la viabilidad celular, capacidad de diferenciación y función es necesario para aplicaciones clínicas e investigación. El objetivo de este estudio fue evaluar si las células troncales de pulpa dental humana (hDPSCs) aisladas y criopreservadas durante 1, 7 y 30 días conservan la viabilidad y expresión de marcadores específicos de células troncales pos crio-preservación. Para esto, las hDPSCs se aislaron de 23 pacientes sanos entre 18 y 31 años. La pulpa dental se disoció enzimáticamente, y las células CD105+ se separaron mediante el sistema Miltenyi™. Posteriormente, las hDPSCs se criopreservaron utilizando el método de Kamath y de Papaccio, se evaluó la viabilidad pos crio-preservación por citometría de flujo (7AAD) y la expresión de marcadores CD105+/ CD73+, CD34-/CD45-. El método de Papaccio, mostró mayor viabilidad celular a los 30 días (59,5%) comparado con el método de Kamath, a 1 día (65,5%) y 7 días (56%) respectivamente. Se observó mayor expresión de los marcadores CD105+/CD34- a 1 y 7 días pos-criopreservación con el método Kamath y CD105+/CD45- a los 3 tiempos de criopreservación. CD73+ presentó mayor expresión en las hDPSCs a las 24 horas y 7 días con el método de Kamath, y al mes con el método Papaccio.

Estos resultados sugieren que las hDPSCs expresan marcadores de células troncales mesenquimales postcriopreservación. Sin embargo el tiempo de criopreservación podría modificar la expresión de los marcadores probablemente por alterar la configuración espacial de las proteínas de membrana celular; o por comprometer a las células a cierto grado de diferenciación.

Palabras clave: *Criopreservación, Células troncales mesenquimales, Fenotipo, Viabilidad celular, Pulpa dental, Medicina regenerativa.*

INTRODUCCIÓN

The discovery of stem cells and their potential has led to the development of new cell therapy strategies[1,2]. The availability and cryopreservation of stem cells have thus become the subjects of intensive research.

Human dental pulp stem cells (hDPSCs) have high potential for proliferation, self-renewal and

differentiation into odontoblasts, osteoblasts, chondrocytes, myocytes, adipocytes, neurons and corneal epithelial cells[1,3,4,5]. Moreover, the ease of obtaining healthy temporary or permanent teeth when they are extracted for treatments such as orthodontics makes dental stem cells an attractive source for autologous transplant as well as for the creation of specialized biobanks providing a reliable supply for applications in regenerative medicine and dentistry. Methods should be developed for storing hDPSCs which involve minimum loss of cell viability and preserve their differentiation potential and function.

The success of hDPSC isolation declines over time. When cell cultures are kept for a long time, certain complications and limitations may arise, such as 1) reduction in their differentiation potential, 2) senescence and apoptosis as a result of serial passages, 3) potential genetic alterations and 4) high costs[5,6]. These are the reasons why cryopreservation is proposed as the most effective method for maintaining cells available in the long term for future clinical applications in transplants[7,8,9]. However, the effects of cryopreservation on the properties of human dental pulp stem cell (hDPSCs) are not clear. Zhang et al.[10] reported that after being stored in liquid nitrogen for two years, hDPSCs maintain their potential for proliferation and differentiation into specific cell lineages[1] although the post-cryopreservation hDPSC isolation success rate may decline due to the formation of ice crystals during the freezing process. The use of cryoprotectants such as dimethyl sulfoxide (DMSO) is therefore suggested to prevent crystals formation[11].

The cryopreservation process may cause cell damage which could be associated to cryoprotectant toxicity or osmotic imbalance upon subjecting the cryoprotectant to freezing and thawing. Several studies have thus related the stress factors in cryopreservation to the activation of cell death signaling cascades[7]. DMSO is the most frequently used cryoprotectant in different methods of cryopreservation. It penetrates cells and forms hydrogen bonds with the water molecules, preventing the flow of water from the cytoplasm, and minimizing dehydration and formation of intracellular ice. However, the most effective cryopreservation protocol for maintaining post-cryopreservation cell viability has not yet been established. Thus, the aim of this study was to evaluate the effect of two cryopreservation methods, one reported by Papaccio *et al.* [12] and the other by Kamath[13], which use the same cryoprotectant (DMSO), to assess the viability and phenotype of mesenchymal stem cells obtained from human dental pulp after undergoing cryopreservation for 1, 7 and 30 days. Selecting the best method for maintaining these hDPSC features is important for their use in biobanks in order to ensure the quality of cryopreserved cells.

MATERIALS AND METHODS

Collection and transportation of extracted teeth

Extracted teeth were obtained from the Dental Clinic at El Bosque University from patients aged 18 to 31 years, with tooth extraction indicated for therapeutic purposes such as included or impacted third molars, or for orthodontic purposes, without caries or periodontal disease. The study was approved by the institution's research ethics committee, and prior informed consent was obtained from the patients. Immediately after extraction, the teeth were placed in sterile phosphate buffered saline (PBS) and transported in ice to the laboratory for processing.

Human dental pulp stem cell (hDPSC) processing and culture

To obtain the dental pulp, the teeth were disinfected with sodium hypochlorite followed by several rinses in sterile phosphate buffered saline (PBS). A high-speed rotary instrument with a sterile Zekrya bur was used to cut them at the cementoenamel junction under constant manual irrigation with sterile PBS. The crown portion was removed manually once the pulp chamber had been approached in order to avoid damaging the tissue. The tissue was placed in DMEM culture medium supplemented with 100U/mL penicillin, 100 µg/mL streptomycin and 2.5 µg/mL amphotericin. Then the tissue was enzymatically dissociated in a solution of 3mg/mL collagenase and 4mg/mL dispase. Cell number and viability were checked in a Neubauer chamber using trypan blue exclusion test. After obtaining an average $1x10^7$ cells, they were subjected to magnetic separation using Miltenyi MiniMACS system and CD105+ antibody conjugated magnetic microbeads, following the manufacturer's instructions. The cell fraction that passed through the column was cultured for use as negative control in flow cytometry tests. The eluted fraction was the enriched fraction of CD105+ hDPSCs. The CD105+ hDPSCs were cultured in 24-

well dishes according to experimental design (Table 1), and kept in NH CFU-F medium (Miltenyi Biotec) at 37°C and 5% CO_2 until 70% cell confluence was achieved.

HDPSC cryopreservation

Once cell confluence was obtained, the hDPSCs were cryopreserved (2.5x10^6 cells) for 1, 7 and 30 days using Method 1, reported by Papaccio et al.[12]: 10% DMSO + 90% fetal bovine serum (FBS), and Method 2, adapted from Kamath *et al.*[13]: 10% DMSO + 70% FBS + 20% NH Stem cells Medium.

Evaluation of hDPSC viability and phenotype after cryopreservation

To determine the efficiency of the cryopreservation protocols, following the freezing times, the cells were thawed in a water bath at 37°C, followed by centrifugation at 2000 rpm. Cell viability was evaluated using flow cytometry with 7-AAD (7 Aminoactinomycin D), a stain which is excluded by viable cells and has high affinity for DNA in dead cells, and phenotype markers expression CD105+/CD73+/CD34-/CD45- in the FACS CANTO II flow cytometer (Becton & Dickinson). These markers are used to characterize mesenchymal stem cells according to the consensus of the International Society for Cellular Therapy (ISCT)[14]. When the culture reached 90% confluence after cryopreservation, the hDPSCs were rinsed in phosphate buffered saline (PBS), trypsin was added at a concentration of 0.25%, and they were centrifuged at 1,500g for 4 minutes, after which the cells stained with trypan blue were counted in inverted microscope with Neubauer chamber. Four Eppendorf tubes were used for the experiment: (1) contained 2.5 x 10^5 unmarked cells, (2) was isotope control containing CD105 marked with phycoerythrin (PE) conjugated with IgG1 (MACS Miltenyi Biotec) and CD45 marked with fluorescein isothiocyanate (FITC) marked with IgG2 (MACS Miltenyi Biotec), (3) contained 2.5 x 10^5 cells with CD105 antibodies marked with PE conjugated with IgG1 (MACS Miltenyi Biotec), CD34 marked with FITC conjugated with IgG2a (MACS Miltenyi Biotec), CD45 marked with peridinin chlorophyll protein (PERCP) conjugated with IgG2a (MACS Miltenyi Biotec); and (4) contained 2.5 x 10^5 cells with the CD73 antibody marked with (PE) conjugated with IgG1 (MACS Miltenyi Biotec) and 7-AAD. They were all incubated for 2 hours before reading in the flow cytometer.

Statistical Analysis

The T-test was used for parametric data and U-Mann Whitney test for non-parametric data. A value of $p \leq 0.05$ was considered statistically significant.

RESULTS

A heterogeneous hDPSCs cell population was obtained from extracted human dental pulp subjected to enzymatic dissociation. The heterogeneous cell suspension was then subjected to magnetic separation using Miltenyi MINIMACS technology to isolate hDPSC CD105+ cells. Under inverted microscope, the cells obtained in fraction CD105+ (hDPSCs) were fibroblast-like, non-refringent and had well-defined spherical nuclei (Fig. 1A). Fibroblast colony-forming units (CFU-F) typical of mesenchymal stem cells were also observed (Fig. 1B). The cells obtained in fraction CD105- were fusiform, fibroblast-like, with cytoplasmic prolongations. The nucleus/cytoplasm ratio was 1/3, suggesting that they may be fibroblasts (Fig. 1C).

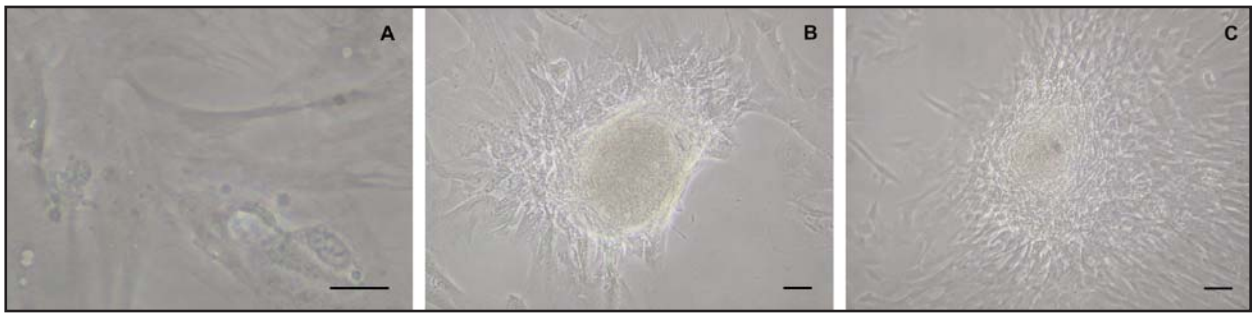

Fig. 1: In vitro morphology of cells obtained by magnetic separation using the Miltenyi system. A. Pulp fibroblasts of the CD 105- fraction (20x). B. Human dental pulp stem cells hDPSC CD105+ (40x). C. hDPSC CD 105+ colony forming unit (20x). Bar 20 μm.

Comparison of the number of viable cells before and after cryopreservation showed that in spite of the fact that viability declines with freezing time, the Kamath method provided better results for number of viable cells (Table 1).

The viability of hDPSC cells after cryopreservation was evaluated by flow cytometry using the fluorescent 7AAD stain. Unstained cells (7AAD-) are viable and stained cells (7AAD+) are dead. For cells that were cryopreserved for 30 days, the Papaccio method showed greater percentage of viability (59.5%) than the Kamath method (56.2%). Nevertheless, the Kamath method provided better viability results for cells cryopreserved for 1 and 7 days (65.5% and 56%, respectively) (Fig. 2).

With regard to immunophenotyping by flow cytometry, for the Papaccio method we observed that cells show greater expression of the markers CD105+/CD34- after 1 and 7 days of freezing (99.9%) than after 30 days (89%). The values for the Kamath method (95.8%, 97.8% and 94.5% after 1, 7 and 30 days, respectively) differed significantly ($p = 0.05$; Fig.3). There was greater expression of CD105+/CD45- with the Papaccio method for all 3 cryopreservation times (95.4%, 96% and 93.2%, respectively) than with the Kamath method (93.5%, 94.2% and 81.3%, respectively). The differences were significant at $p = 0.05$ (Fig.3). Moreover, post-cryopreservation hDPSCs did not express the markers CD34-/CD45-, suggesting that they are not hematopoietic stem cells.

The CD73 marker was found to have less expression after 1 day with both cryopreservation methods, with 84% and 79.6%, respectively. At 7 days they were 97.6% and 94.8% respectively, and at 30 days, expression declined to 72.8% with the Papaccio method, in contrast to 94.8% with the Kamath method ($p= 0.05$; Fig.3).

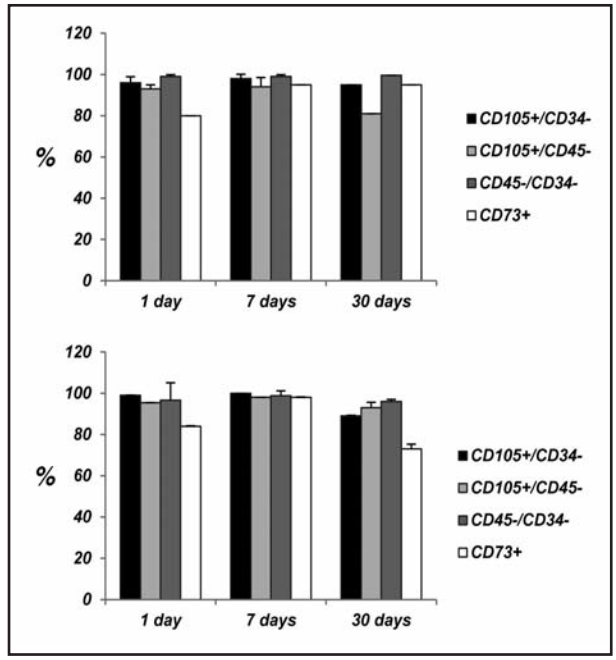

Fig. 2: Percentage of post-cryopreservation hDPSC which express the phenotypes CD105+/CD34-, CD105+/CD45-, CD45-/CD34- and CD73+. A. Kamath cryopreservation method, B. Papaccio cryopreservation method (p= 0.005).

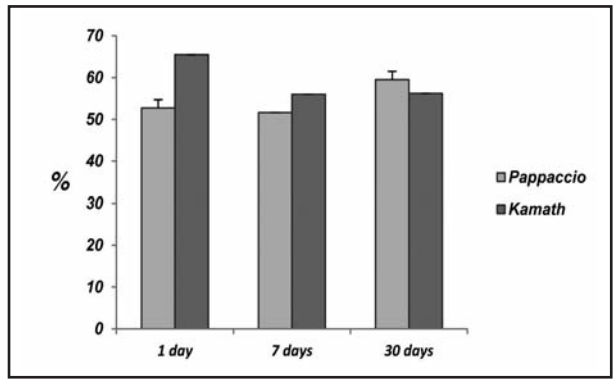

Fig. 3: Percentage of viable post-cryopreservation hDPSC using 7AAD dye-exclusion stain, for the Papaccio and Kamath methods.

Table 1: Cell counting ofhDPSCwithout cryopreservation and following the postcryopreservation methods (n=18).

	Kamath Method		Papaccio Method	
Cryopreservation time	**Average ± SDM without cryopreservation**	**Average ± SDM with cryopreservation**	**Average ± SDM without cryopreservation**	**Average ± SDM with cryopreservation**
1 day	266500 ± 143411	165417 ± 126709	207500 ± 38891	80000 ± 0
7 days	305833 ± 155168	221417 ± 96981	165000 ± 21213	75000 ± 35355
30 days	378000 ± 211644	3144 ± 1596	963750 ± 1253347	381 ± 539

Data are expressed as means +/-standard deviation

DISCUSSION

Freezing mesenchymal stem cells in stable state is an essential requisite for their application in tissue engineering and regenerative medical applications. The standardization of cryopreservation protocols for these cells has enabled the creation of banks to make them available for future applications in transplants or therapies required to ensure patient survival.

The purpose of cryopreservation is to reduce cell metabolic activity for long periods of time while at the same time preserving viability, phenotype and differentiation potential[9]. However, it may induce alterations in chemical, thermal and electrical properties of cell membranes, organelles and molecular structures involved in cell-cell/cell-extracellular matrix interactions.

In this study, mesenchymal stem cells were obtained from human dental pulp from permanent teeth. It was evaluated whether they preserve their characteristic phenotype and viability after cryopreservation in liquid nitrogen for 1, 7 and 30 days, comparing the Papaccio method[12] (10% DMSO and 90% FBS) and the commercial Kamath method[13] (10% DMSO, 70% FBS and 20% culture medium).

Ideal cryopreservation and subsequent recovery of viable cells depend on various parameters: (1) the processing time and storage temperature after isolation; (2) the sampling protocol; (3) the cryopreservant selected; (4) the freezing and thawing protocols and (5) the long-term storage temperatures[8].

In this study, the pulp tissue samples were transported at 4°C in DMEM culture medium supplemented with 100U/mL penicillin / 100 µg /mL streptomycin and 2.5 µg/mL amphotericin to eliminate any contaminant proceeding from the oral cavity[14]. The pulp tissue was processed for cell culture within 3 hours after extraction. In a previous study, Temmerman*y* et al. stored the tissue after extraction for 24h at 4°C in culture medium supplemented with antibiotics[12], which might have reduced the recovery of viable tissue cells[15].

The hDPSCs were obtained from dental pulp by enzymatic dissociation with collagenase/dispase for 2 hours with constant stirring at 37ºC, as reported by Woods et al.[9] It has been suggested that this procedure may compromise the integrity of cell membranes[8]. Although explant culture is more convenient than enzymatic digestion, the cells migrate slowly from the tissue fragments until they attain confluence after approximately 2-3 weeks[16]. Moreover, enzymatic digestion enables CFU-F colony-forming units to be obtained after 1 - 2 weeks in culture, making it the most efficient method[17,18].

Gronthos et al. and Batouli et al. used the enzymatic digestion method and showed that hDPSCs differentiate into odontoblast-like cells which form the dentin matrix *in vivo,* suggesting that this method does not alter the differentiation potential of stem cells[18,19]. In contrast, it was shown that with the explant culture method, cells are potentially able to differentiate into odontoblasts and form mineralized nodules *in vitro*[20-23]. To conclude, enzymatic digestion is an efficient method for isolating hDPSCs which meet the typical criteria for postnatal somatic stem cells[24], such as high proliferation rate, clonogenic nature and co-expression of specific markers[18]. In our study, enzymatic digestion and subsequent magnetic separation did not affect cell confluence, which was achieved at 2 weeks in culture, compared to the study by Temmerman et al.,[15] where confluence was achieved at 23 days. In addition, colony-forming units (CFU-F) were observed, as reported by Polisetty et al.[25]

Cryopreservation is the process of cooling and storing cells, tissues or organs at temperatures beneath -80°C, and usually as cold as −196°C, to maintain their viability[26]. The cooling process involves complex phenomena of water crystallization and changes in intracellular and extracellular solute concentrations, which may be harmful to cell survival. In addition, it has been reported that cell exposure to low temperatures induces stress leading to cell death[26 - 28].

The main steps for the cryopreservation of most cell types are usually: (1) isolating cells, (2) adding cryopreservant, (3) inducing ice crystals in the cell suspension after a certain cooling rate (-1 to -10ºC/min), (4) long-term storage at cryogenic temperatures (usually in liquid nitrogen), (5) rapid thawing at 37ºC, (6) removal of cryoprotectant by centrifuging, and (7) seeding cells to enable their growth in culture[29, 30].

Cell damage due to cryopreservation may be due to a combination of the following: (1) cryoprotectant toxicity[31,32]; (2) osmotic damage due to exposure

of cryoprotectants to freezing-thawing[29,33]; (3) formation of intracellular ice during cooling[33] and (4) re-crystallization of intracellular ice during warming[34]. Several studies have associated cryopreservation stress factors to the activation of cell death signaling cascades[26].

Conventional techniques for freezing stem cells, which include slow freezing, rapid thawing and vitrification have proved refractory for these cells, which exhibit low survival rates[35].

The low efficiency of stem cells to cryopreservation has been partly attributed to the fact that they need to be in close physical contact with one another within the colony to enable cell-cell signaling[36]. These observations show that this type of cell is highly sensitive to cryopreservation.

Heng et al. suggested for the first time that apoptosis, rather than necrosis, is the primary mechanism for the loss of viability in human embryonic stem cells during freezing/thawing with conventional slow cooling protocols[37]. They showed that most cells (~98%) are viable immediately after thawing (determined by trypan blue exclusion) and that cell viability declines gradually over time in culture at 37ºC[38]. DMSO (Me2SO) has been used as a cryopreservant for hematopoietic stem cells (HCP)[39] and is currently used in conventional protocols. For therapeutic stem cell applications, cryopreservation is performed immediately after isolation[40], using 1–2 M DMSO concentrations in a freezer with a controlled cooling rate of -1 °C / min, storage and/or transportation below -135°C, preferably between -150 and -196°C, and rapid thawing at 37°C[41–45]. The DMSO penetrates the cells and forms hydrogen bonds with the water molecules, blocking the flow of water from the cytoplasm and minimizing dehydration and the formation of intracellular ice during cryopreservation[46-49]. In addition, both the bovine fetal serum and the NH culture medium in the Papaccio and Kamath methods may protect cells from oxygen free radicals which are formed during freezing[47].

In this regard, it has been noted that cryopreservation can in fact compromise hDPSC viability, regardless of the freezing method used. However, it has been observed that the Kamath method provides higher values for cell viability on the first and seventh days (65.5% and 56%, respectively), while the Papaccio method does so at 30 days of freezing (59.5%) (Fig.3). Woods et al.[9] determined that dimethyl sulfoxide at concentrations of 1 to 1.5 M is a better cryoprotectant than ethylene glycol and propylene glycol at the same concentrations. In fact, this concentration is equivalent to the 10% DMSO employed in both cryopreservation methods used in this study.

With regard to the expression of stem cell markers, it was found that the cells preserve their undifferentiated cell phenotype regardless of the freezing method. However, a reduction in the expression of CD73+ was observed after 7 and 30 days with both freezing methods.

The CD73 marker or 5'-ectonucleotidase is considered as a lineage marker for mesenchymal stem cells and is believed to be related to cell adhesion mechanisms, because it has been found co-expressed with integrin α2 type molecules, which has characterized CD73 as a cell adhesion mediator in MSCs. The presence of CD73- cells suggest that they lose their capacity to differentiate into cardiac myocytes, although they do keep their potential to differentiate into adipocytes and osteoblasts *in vitro*[49, 50.]

These results suggest that although hDPSCs lose cell viability after cryopreservation, they keep mesenchymal stem cell marker expression. However, this expression depends on freezing time, probably due to the alteration of the 3-D shape of cell membrane proteins or because cells are compromised at a certain level of differentiation.

ACKNOWLEDGMENTS

This study is part of to research project PCI 2010-142, funded by internal call for entries at the Research Department of El Bosque University

CORRESPONDENCE

Dr. Juan Carlos Munévar
Universidad El Bosque
Unidad de Investigación Básica Oral. 2 piso.
División de Investigaciones.
Avenida Carrera 9 No 131ª – 02.Código Postal 110121,
Bogotá, Colombia
munevarjuan@unbosque.edu.co

REFERENCES

1. Brar GS, Toor RS. Dental stem cells: dentinogenic, osteogenic, and neurogenic differentiation and its clinical cell based therapies. Indian J Dent Res 2012; 23:393-397.
2. Rodríguez-Lozano FJ, Insausti CL, Iniesta F, Blanquer M, Ramírez MC, Meseguer L, Meseguer-Henarejos AB, Marín N, et al. Mesenchymal dental stem cells in regenerative dentistry. Med Oral Patol Oral Cir Bucal 2012; 17:e1062-1067.
3. Akiyama K, Chen C, Gronthos S, Shi S. Lineage differentiation of mesenchymal stem cells from dental pulp, apical papilla, and periodontal ligament. Methods Mol Biol 2012; 887:111-121.
4. Kawashima N. Characterisation of dental pulp stem cells: a new horizon for tissue regeneration? Arch Oral Biol 2012; 57:1439-1458.
5. Estrela C, Alencar AH, Kitten GT, Vencio EF, Gava E. Mesenchymal Stem Cells in the Dental Tissues: Perspectives for Tissue Regeneration. Braz Dent J 2012; 22:91-98.
6. Martin-Piedra MA, Garzon I, Oliveira AC, Alfonso-Rodriguez CA, Carriel V, Scionti G, et al. Cell viability and proliferation capability of long-term human dental pulp stem cell cultures. Cytotherapy 2014; 16:266-277.
7. Lee SY, Chiang PC, Tsai YH, Tsai SY, Jeng JH, Kawata T, Huang HM. Effects of cryopreservation of intact teeth on the isolated dental pulp stem cells. J Endod. 2010; 36: 1336-1340.
8. Freshney RI: Cryopreservation. In: Freshney RI: Culture of animal cells a manual of basic technique. New York, New York, USA: Wiley-Liss Inc, 2000: 297-308.
9. Woods EJ, Benson JD, Agca Y, Critser JK. Fundamental cryobiology of reproductive cells and tissues. Cryobiology 2004; 48:146-156.
10. Zhang X, Mitsuru A, Igura K, Takahashi K, Ichinose S, Yamaguchi S, Takahashi TA. Mesenchymal progenitor cells derived from chorionic villi of human placenta for cartilage tissue engineering. Biochem Biophys Res Commun 2006; 340:944-952.
11. Zhang W, Walboomers XF, Shi S, Fan M, Jansen JA. Multilineage differentiation potential of stem cells derived from human dental pulp after cryopreservation. Tissue Eng Part C 2006; 12:2813-2823.
12. Papaccio G, Graziano A, d´Aquino R, Graziano MF, Pirozzi G, Menditti D, De Rosa A, Carinci F, et al. Long-term Cryopreservation of dental pulp stem cells (SBP-DPSCs) and their differentiated osteoblasts: a cell source for tissue repair. J Cell Physiol 2006; 208:319-325.
13. Kamath A. Human Mesenchymal Stem Cell Protocol: cryopreservation. SC Protocol Sheet: 00007. Cellular Engineering Technologies, Inc. Thermo Fisher Scientific Inc. 2007.
14. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006; 8:315-317.
15. Temmerman L, Beele H, Dermaut LR, Van Maele G, De Pauw GA. Influence of cryopreservationon the pulpal tissue of immature third molars in vitro.Cell Tissue Bank 2010; 11:281-289.
16. Seo BM., Miura M, Sonoyama W, Coppe C, Stanyon R, Shi S. Recovery of stem cells from cryopreserved periodontal ligament. J Dent Res 2005; 84:907-912.
17. Huang GT, Sonoyama W, Chen J, Park SH. In vitro characterization of human dental pulp cells: various isolation methods and culturing environments. Cell tissue Res 2006; 324:225-236.
18. Gronthos S, Mankani M, Brahim J, Robey PG, Shi S. Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. Proc Natl Acad Sci USA 2000; 97:13625-13630.
19. Batouli S, Miura M, Brahim J, Tsutsui TW, Fisher LW, Gronthos S, Robey PG, Shi S. Comparison of stem-cell-mediated osteogenesis and dentinogenesis. J Dent Res 2003; 82:976-981.
20. About I, Bottero MJ, de Denato P, Camps J, Franquin JC, Mitsiadis TA. Human dentin production in vitro. Exp Cell Res 2000; 258:33-41.
21. Shiba H, Fujita T, Doi N, Nakamura S, Nakanishi K, Takemoto T, Hino T, Noshiro M, et al. Differential effects of various growth factors and cytokines on the syntheses of DNA, type I collagen, laminin, fibronectin, osteonectin/ secreted protein, acidic and rich in cysteine (SPARC), and alkaline phosphatase by human pulp cells in culture. J Cell Physiol 1998; 174:194-205.
22. Papagerakis P, Berdal A, Mesbah M, Peuchmaur M, Malaval L, Nydegger J, Simmer J, Macdougall M. Investigation of osteocalcin, osteonectin, and dentin sialophosphoprotein in developing human teeth. Bone 2002; 30:377-385.
23. Couble ML, Farges JC, Bleicher F, Perrat-Mabillon B, Boudeulle M, Magloire H. Odontoblast differentiation of human dental pulp cells in explant cultures. Calcif Tissue Int 2000; 66:129-138.
24. Robey PG. Series Introduction: Stem cells near the century mark. J Clin Invest 2000; 105:1489-1491.
25. Polisetty N, Fatima A, Madhira SL, Sangwan VS, Vemuganti GK. Mesenchymal cells from limbal stroma of human eye. Mol Vis 2008; 14:431-442.
26. Baust JG, Gao D, Baust JM. Cryopreservation: An emerging paradigm change. Organogenesis 2009; 5:90-96.
27. Paasch U, Sharma RK, Gupta AK, Grunewald S, Mascha EJ, Thomas AJ Jr, Glander HJ, Agarwal A. Cryopreservation and thawing is associated with varying extent of activation of apoptotic machinery in subsets of ejaculated human spermatozoa. Biol Reprod 2004; 71:1828-1837.
28. Xiao M, Dooley DC. Assessment of cell viability and apoptosis in human umbilical cord blood following storage. J Hematother Stem Cell Res 2003; 12:115-122.
29. Gao DY, Chang Q, Liu C, Farris K, Harvey K, McGann LE, English D, Jansen J, et al. Fundamental cryobiology of human hematopoietic progenitor cells. I: Osmotic characteristics and volume distribution. Cryobiology 1998; 36:40-48.
30. Hubel A. Parameters of cell freezing: implications for the cryopreservation of stem cells. Transfus Med Rev 1997; 11:224-233.
31. Muldrew K, McGann LE. The osmotic rupture hypothesis of intracellular freezing injury. Biophys J 1994; 66:532-541.
32. Schneider U, Maurer RR. Factors affecting survival of frozen-thawed mouse embryos. Biol Reprod 1983; 29:121-128.

33. Mazur P, Schneider U. Osmotic responses of preimplantation mouse and bovine embryos and their cryobiological implications Cell Biophys 1986; 8:259-285.
34. Mazur P, Cole KW. Roles of unfrozen fraction, salt concentration, and changes in cell volume in the survival of frozen human erythrocytes. Cryobiology 1989; 26:1-29.
35. Richards M, Fong CY, Tan S, Chan WK, Bongso A. An efficient and safe xeno-free cryopreservation method for the storage of human embryonic stem cells. Stem Cells 2004; 22:779-789. Erratum in: Stem Cells. 2005; 23:604.
36. Reubinoff BE, Pera MF, Vajta G, Trounson AO. Effective cryopreservation of human embryonic stem cells by the open pulled straw vitrification method. Hum Reprod 2001; 16:2187-2194.
37. Heng BC, Ye CP, Liu H, Toh WS, Rufaihah AJ, Cao T. Kinetics of cell death of frozen-thawed human embryonic stem cell colonies is reversibly slowed down by exposure to low temperature. Zygote 2006; 14:341-348.
38. Liu Y, Xu X, Ma X, Martin-Rendon E, Watt S, Cui Z. Cryopreservation of human bone marrow-derived mesenchymal stem cells with reduced dimethylsulfoxide and well-defined freezing solutions. Biotechnol Prog 2010; 26:1635-1643.
39. Guttridge, M. G, Sidders, C, Booth-Davey, E, Panaphilor, D, and Watt, S. M. Factors affecting volume reduction and red blood cell depletion of bone memous on the cobe spectra separator prior to haematopoietic stem cell transplantation. Bone Marrow Transplant 2006; 38:175-181.
40. Hunt, C. J, Armitage, S. E, and Pegg, D. E. Cryopreservation of umbilical cord blood: 2. Tolerance of CD34(+) cells to multimolar dimethyl sulphoxide and the effect of cooling rate on recovery after freezing and thawing. Cryobiology 2003; 46: 76-87.
41. Hunt, C. J, Armitage, S. E, and Pegg, D. E. Cryopreservation of umbilical cord blood: 1. Osmotically inactive volume, hydraulic conductivity and permeability of CD34(+) cells to dimethyl sulphoxide. Cryobiology 2003; 46:61-75.
42. Yang, H, Zhao, H, Acker, J. P, Liu, J. Z, Akabutu, J, and McGann, L. E. Effect of dimethyl sulphoxide on post-thaw viability assessment of CD45+ and CD34+ cells of umbilical cord blood and mobilized peripheral blood. Cryobiology 2005; 51:165-175.
43. Sartor, M, Antonenas, V, Garvin, F, Webb, M, and Bradstock, K. F. Recovery of viable CD34+ cells from cryopreserved hematopoietic progenitor cell products. Bone Marrow Transplant 2005; 36:199-204.
44. Ojeda-Uribe, M, Sovalat, H, Bourderont, D, Brunot A, Marr A, Lewandowski H, Chabouté V, Peter P,et al. Peripheral blood and BM CD34+ CD38- cells show better resistance to cryopreservation than CD34+ CD38+ cells in autologous stem cell transplantation. Cytotherapy2004; 6:571-583.
45. LovelockJE, Bishop M.W. Prevention of freezing damage to living cells by dimethyl sulphoxide. Nature 1959; 183: 1394-1395.
46. Gutteridge JM, Quinlan G.J. Antioxidant protection against organic and inorganic oxygen radicals by normal human plasma: the important primary role for iron-binding and iron-oxidising proteins. BiochimBiophys Acta 1993; 1156: 144-150.
47. Windrum P, Morris TC, Drake MB, Niederwieser D, Ruutu T. EBMT Chronic Leukaemia Working Party Complications Subcommittee. Variation in dimethyl sulfoxide use in stem cell transplantation: a survey of EBMT centres. Bone Marrow Transplant 2005; 36:601-603.
48. Mandumpal JB, Kreck CA, Mancera RL.A molecular mechanism of solvent cryoprotection in aqueous DMSO solutions. Phys Chem Chem Phys 2011; 13:3839-3842.
49. Airas L, Niemelä J, Salmi M, Puurunen T, Smith DJ, Jalkanen S. Differential regulation and function of CD73, a Glycosyl-Phosphatidylinositol-linked 70-kD adhesion molecule, on lymphocytes and endothelial cells. J Cell Biol 1997; 136:421-431.
50. Sträter N. Ecto- 5'-nucleotidase: Structure function relationships. Purinergic Signalling 2006; 2:343-350.