Original Research
Pulp Biology

Proliferation rate and expression of stem cells markers during expansion in primary culture of pulp cells

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Abstract: The aim of the present study was to evaluate the proliferation rate and the expression of stem cells markers during expansion in primary culture of dental pulp stem cells (DPSCs), comparing different techniques (explant and enzymatic digestion), subject ages (up to 40 and over 40) and cell passages (#2, #5 and #8). DPSCs were isolated using either the enzymatic digestion (ED) or explant (EX) technique. The number of days needed for the cells to reach confluence was determined. Immunophenotyping was performed by immunofluorescence and flow cytometry analysis using antibodies specific for nestin, vimentin, CD44, CD146, Oct3/4 and CD34. Data were subjected to three-way analysis of variance (n = 6/group). The ANOVA tests were complemented by Tukey's or t-tests ($p < 0.05$). The variables "donor age" and "technique" were analyzed to define the optimal desirability value using a response optimization. DPSCs presented a high proliferation rate from passages 2 to 5 while cells from passage 8 proliferated at a slower rate. For all markers, no significant difference was observed among passages, irrespective of the technique used or the donor's age. The mean fraction of specific antibodies was 73.7% (± 11.5), 49.0% (± 18.7), 80.1% (± 8.0), 45.2% (± 13.7), 64.7% (± 5.3) and 2.0% (± 1.5) for CD44, OCT, vimentin, nestin, CD146 and CD34, respectively. The highest optimal desirability value was obtained using the ED technique and cells from younger patients (d = 0.92). However, it was concluded that neither the isolation technique nor the donor age or cell passage significantly interfered with the stem cell phenotype and proliferation rate during cell expansion.

Keywords: Dental Pulp; Immunophenotyping; Stem Cells.

Introduction

Tissue bioengineering is a field in which research projects are constantly being conducted and it has shown notable evolution. The development of materials and techniques for tissue regeneration appears to be a potential alternative in the presence of tissue lesions and an attempt to replace the transplantation of organs.[1,2,3] Among the different techniques reported in the literature, one may note the use of some biomaterials, such as hydrogels, scaffolds, microspheres and nanofibers.[4] However, an alternative that has become increasingly investigated is the use of cell sources for tissue regeneration, mainly stem cells.[4,5]

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Stem cells represents an important component of regenerative medicine and tissue engineering since they have the capacity to differentiate into a variety of cell types and have been used for the treatment of several diseases, including ischemia, neuronal degeneration and diabetes in animal models,[6,7,8] as well as in bone regeneration[9]. These cells may be isolated from a number of tissues, including bone marrow, blood, the brain, liver, muscle, skin and dental pulp.[10-13]

Despite the different eligible sources of stem cells, dental pulp has been extensively investigated because it is an easily available tissue and has great capacity for proliferation and differentiation in vitro.[14-16] Stem cells derived from dental pulp may be obtained from healthy permanent teeth (DPSCs) or healthy primary teeth, even at an advanced stage of exfoliation (stem cells of human exfoliated deciduous teeth - SHEDs).[8,17-20] These cells originate from the cranial neural crest and express early markers of mesenchymal cells and neuroectodermal stem cells.[21] Furthermore, they are multipotent and may differentiate into osteoblasts, chondrocytes, adipocytes, endothelial cells and neuronal cells.[22]

Two methods of obtaining stem cells from pulp tissue are reported in the literature.[22,23] The first is tissue explant in which small pieces of pulp tissue are placed in Petri dishes and kept in a culture medium until the beginning of cell migration to the dish.[22] The second is enzymatic digestion in which the application of specific enzymes (collagenase type I and dispase) is necessary for the digestion of tissue components and to obtain primary in vitro cultures.[23]

It is still not known whether cells obtained by these different techniques present some different phenotypic characteristics or whether one technique is more efficient than the other for obtaining stem cells. It is also possible that phenotypic variation may occur according to the type of niche (young or old donors), with the passage or explant in which the cell/tissue is found or even according to the technique used to obtain the cells.

Detailed characterization, comparing the different parameters that may be used for obtaining stem cells, in addition to follow-up of the cell behavior with each passage, is necessary for the more efficient use of these cells in tissue regeneration. The aim of the present study was to evaluate the proliferation rate and the expression of some specific stem cells markers during natural expansion in primary culture of pulp cells obtained from healthy permanent teeth, comparing different techniques (explant and enzymatic digestion), subject ages (up to 40 and over 40) and cell passages (#2, #5 and #8). The null hypothesis was that regardless the age of the donor, cell expansion technique and cell passage, pulp cells would have the same phenotype characteristics and same proliferation rate during natural expansion.

Methodology

Pulp cells isolation techniques

Pulp cells were obtained from six healthy human premolars teeth (three from donors up to 40 years old and three from donors over 40 years old)[24,25] extracted due to orthodontic reasons at the Forsyth Dental Clinic and Harvard School of Dental Medicine under informed consent (IRB 037, IRB 14-1841). All donors were healthy Caucasians, no smokers and did not take any medication. Each age group was comprised of two males, and one female donor. The dental pulp was mechanically removed (after longitudinal odontosection), with dentin spoon excavators (Golgran Ind Instr Odontológicos, São Caetano do Sul, SP, Brazil) according to the size of the pulp chamber, under aseptic conditions and divided into two equal parts, utilizing a surgical scalpel blade nº 15 (Swann-Morton, Sheffield, UK). The cells were isolated using two different techniques:

Enzymatic Digestion (ED): half of the pulp tissue was immersed at 37°C for 24 hours in a solution containing 3 mg/mL collagenase type I and 4 mg/mL dispase (Worthington Biochemical Corp, Lakewood, USA). After this period, the solution containing the remainder of digested pulp structures was centrifuged at 1200 rpm for 2 minutes. The supernatant was discarded and the pellet formed at the bottom of the Falcon tube (TPP Techno Plastic Products AG, Trasadingen, Switzerland) was resuspended in Dulbecco's modified Eagle'smedium (DMEM)/Ham's F12 (DMEM/F12, Invitrogen Corporation, Carlsbad, USA) supplemented with 15% fetal bovine serum

(FBS, Hy Clone, Logan, USA). The cells were seeded in 25 cm^2 culture flasks and incubated at 37°C with 5% CO_2 for 1 day. After this incubation period, the first change of culture medium was made, and the culture medium was replaced two times per week. Subculturing occurred at 80% confluence.

Tissue Explant (EX): the other half of the pulp tissue was gently washed with a buffered phosphate solution (PBS, Invitrogen, Carlsbad, USA) and placed in a 35-mm culture plate (Corning Inc., Corning, USA). The tissue explants were cultivated in DMEM/Ham's F12 supplemented with 15% FBS, 100 units/ml penicillin, 100 mg/ml streptomycin, 2 mM L-glutamine, and 2 mM nonessential amino acids (Invitrogen Corporation, Carlsbad, CA, USA) in a humid atmosphere of 5% CO_2 at 37°C. After cell confluence around the tissue, it was removed and the cells were trypsinized (0.5 g/L trypsin and 0.53 mmol/L ethylenediamine tetra-acetic acid - Invitrogen Corporation, Carlsbad, USA) and transferred to a 25 cm^2 flask.

For both techniques, cells over passage were cultivated in 75 cm^2 flasks. Once the cells achieved 80% confluence, 3/4 of the cells were used for evaluations, and 1/4 were used to expand the next passage. The same procedure was performed until passage 8. During cell expansion, the number of days necessary for the cells to reach confluence was recorded from passage 0 to 8. The aim of this evaluation was to establish the proliferation rate of pulp cells during expansion, considering all different donor ages and techniques.

Immunophenotyping

The immunophenotyping of the pulp cells was based on immunofluorescence and flow cytometry analyses using specific antibodies for mesenchymal stem cell characterization, such as (+) nestin (1:100), vimentin (1:100), CD44 (1:50), CD146 (1:50), Oct3/4 (1:200) and (-) CD34 (1:50) (Abcam, Cambridge, USA). Secondary antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 596 (Life Tech, Carlsbad, USA) were also used (1:500).

For flow cytometry, cells were trypsinized for 5 minutes, centrifuged and washed with 0.1% BSA in 0.1 M PBS at 4°C. The cells were then fixed in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, USA) in PBS, and for cytoplasmic markers, cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, USA) in PBS. After this, the cells (100 cells/mL) were incubated with the primary antibody (50 µl) for 1 hour at room temperature. Cells were washed again and incubated for 45 minutes (dark room) with 50 µl of the secondary antibody solution at room temperature. The cells were then washed three times with PBS and resuspended in 0.25 ml of chilled PBS. The analysis was performed in a flow cytometer (FACS; Becton, Dickinson, San Jose, CA) using the program from Quest CELL (Becton, Dickinson).

For the immunofluorescence analysis, the cells were fixed and permeabilized as described for immunofluorescence. Then, the cells were incubated with 5% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA) diluted in PBS for 30 minutes and incubated at 4°C overnight with the primary antibodies. Later, the cells were washed with PBS and incubated with secondary antibody for 45 minutes (dark room). After that, a 4'6'-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA) solution (1:500) in PBS was applied to the cells for 5 minutes. The microscope slides were mounted in Antifade medium (Life Tech, Carlsbad, USA), and immunofluorescence was detected using a WideField fluorescence microscope (LSM 410, Zeiss, Jena, Germany). The digital images were acquired with a CCD camera (Applied Imaging model ER 339), and the documentation program used was ZEN Blue edition (ZEISS Microscope Software). Cells from passage 2, 5 and 8 were analyzed.

Data analysis

A qualitative interpretation of immunofluorescence and inverted microscope images was performed. Quantitative data obtained by flow cytometry were evaluated using three-way analysis of variance (type of technique, donor age, and number of passages, n = 6/group). The ANOVA tests were complemented by Tukey's or t-tests. All statistical tests were considered at the predetermined level of significance of 5%.

Furthermore, the variables "donor age" and "technique" were analyzed to define the optimal desirability value using a response optimization, considering all positive markers used in this study (Minitab 16.2.4.4- Minitab Inc., State College,

USA). Response optimization helps to identify the combination of variable settings that jointly optimize a single response or a set of responses. The values of desirability functions lie between 0 and 1. The value 0 is attributed when the factors give an unwanted response, while the value 1 corresponds to the optimal performance for the studied factors.[26]

Results

Pulp cells isolation techniques

The number of days necessary to reach confluence considering the donor age and DPSC passage is shown in Figures 1 and 2, respectively for ED and EX. It was observed that DPSCs presented a high proliferation rate from passages 2 to 5, while for passage 8, cells took slightly longer to reach confluence mainly for the 60-year-old donor. The same behavior was observed for both techniques. The most evident difference between techniques was detected for passage 0–1. While cells from the ED technique took 10–13 days to reach confluence, 15–18 days were required when the EX technique was used. For all passages, irrespective of the cell expansion technique, two morphologically different cell types (elongated and circular) were observed in a similar confluence. The niche formation was detected for all passages and techniques (arrows) (Figure 3). Additionally, the aspect of pulp cells for enzymatic digestion (ED) and Explant (EX), 6 and 15 days after tissue obtaining are shown in Figure 4.

Immunophenotyping

Flow cytometry

Figure 5 shows the concentrations (%) of DPSCs positively marked with CD44 (A), OCT (B) and vimentin (C), and Figure 6 with nestin (A), CD146 (B) and CD34 (C) in passages 2, 5 and 8, comparing the cell expansion technique (ED and EX) and donor age (up to age 40 and over 40). For all markers, no significant difference was observed among passages, regardless of the technique used or the donor age. The mean concentration was 73,7% (±11,5) for CD44, 49,0% (±18,7) for OCT, 80,1% (± 8,0) for vimentin, 45,2% (± 13,7) for nestin, 64,7% (±5,3) for CD146 and 2,0% (± 1,5) for CD34.

Response optimizer

Desirability values for each marker separately and all markers together (D value) is shown in Table as a function of DPSC expansion technique and donor age group. The highest value (D = 0.92475) was found using the ED technique and donors aged up to 40 years old. When the donor age group is changed from up to 40 to over 40, the desirability value decreases for OCT (D = 0.63326) and nestin (D = 0.76181), and the overall D value drops to 0.63326. Conversely, desirability values for cells expanded using the EX technique was not affected by the donor age group.

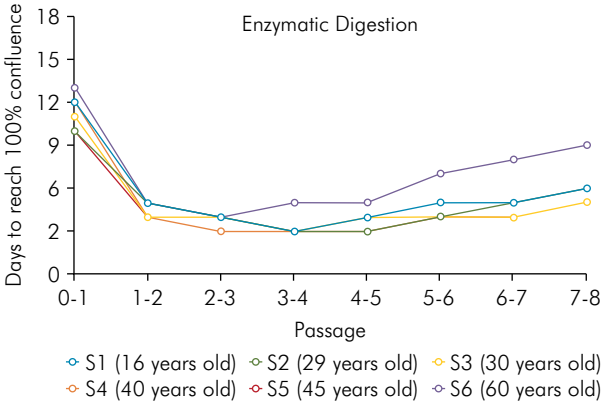

Figure 1. Line graph representative of the days necessary to reach confluence during dental pulp stem cells (DPSC) expansion, considering all donor ages and cell passages, for ENZYMATIC DIGESTION (ED).

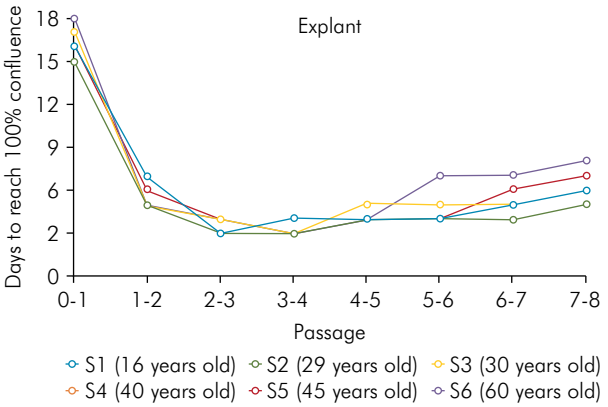

Figure 2. Line graph representative of the days necessary to reach confluence during DPSC dental pulp stem cells (DPSC) expansion, considering all donor ages and cell passages for EXPLANT (EX).

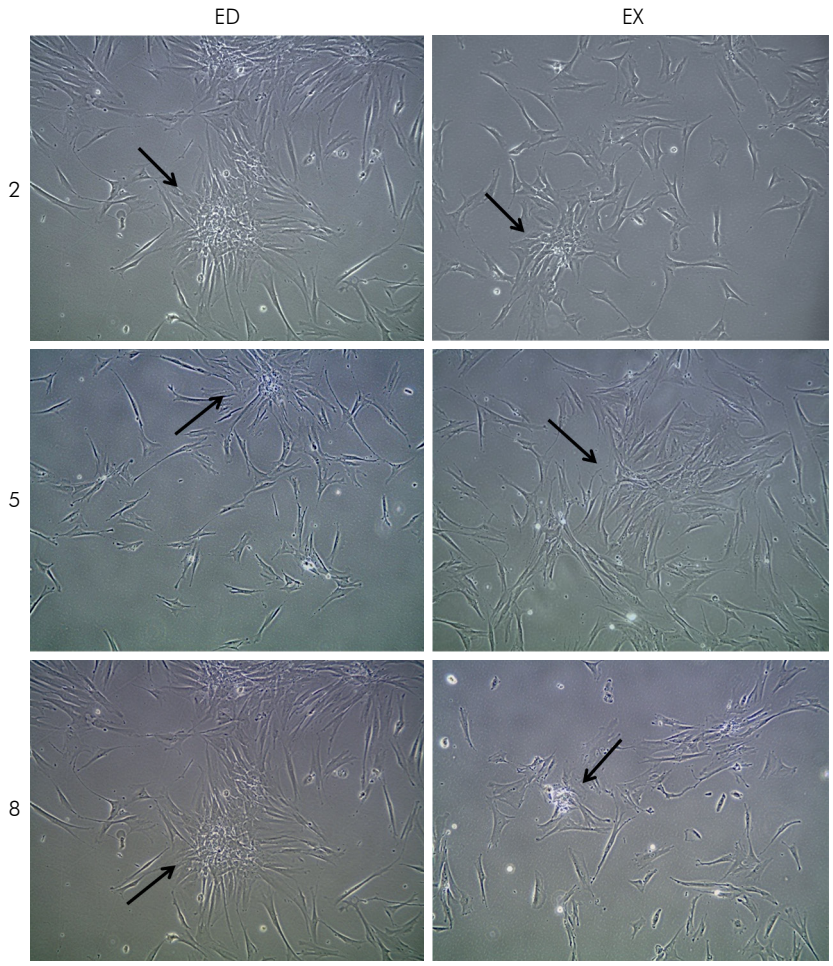

Figure 3. Inverted microscope images (20X) indicating dental pulp stem cells (DPSC)morphology and confluence, considering the techniques enzymatic digestion (ED) and explant (EX) and passages (2, 5 and 8), on the day after seeding. Arrows indicate multicellular nodules formation.

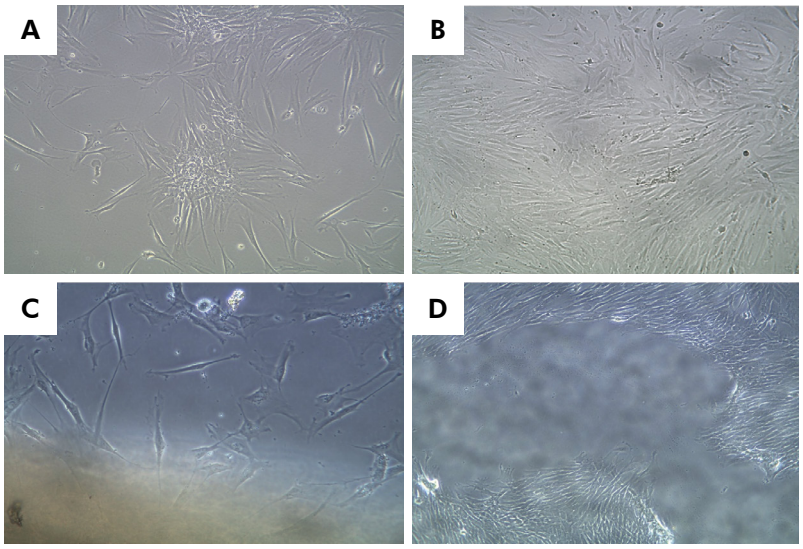

Figure 4. Aspect of pulp cells for enzymatic digestion (ED) technique (A and B) and Explant (EX) technique (C and D). (A) – 6 days after tissue obtaining, (B) –15 days of tissue obtaining, (C) – 6 days after tissue obtaining, (D) - 15 days of tissue obtaining. 29-year-old donor. A, B and D – X10, C- X40.

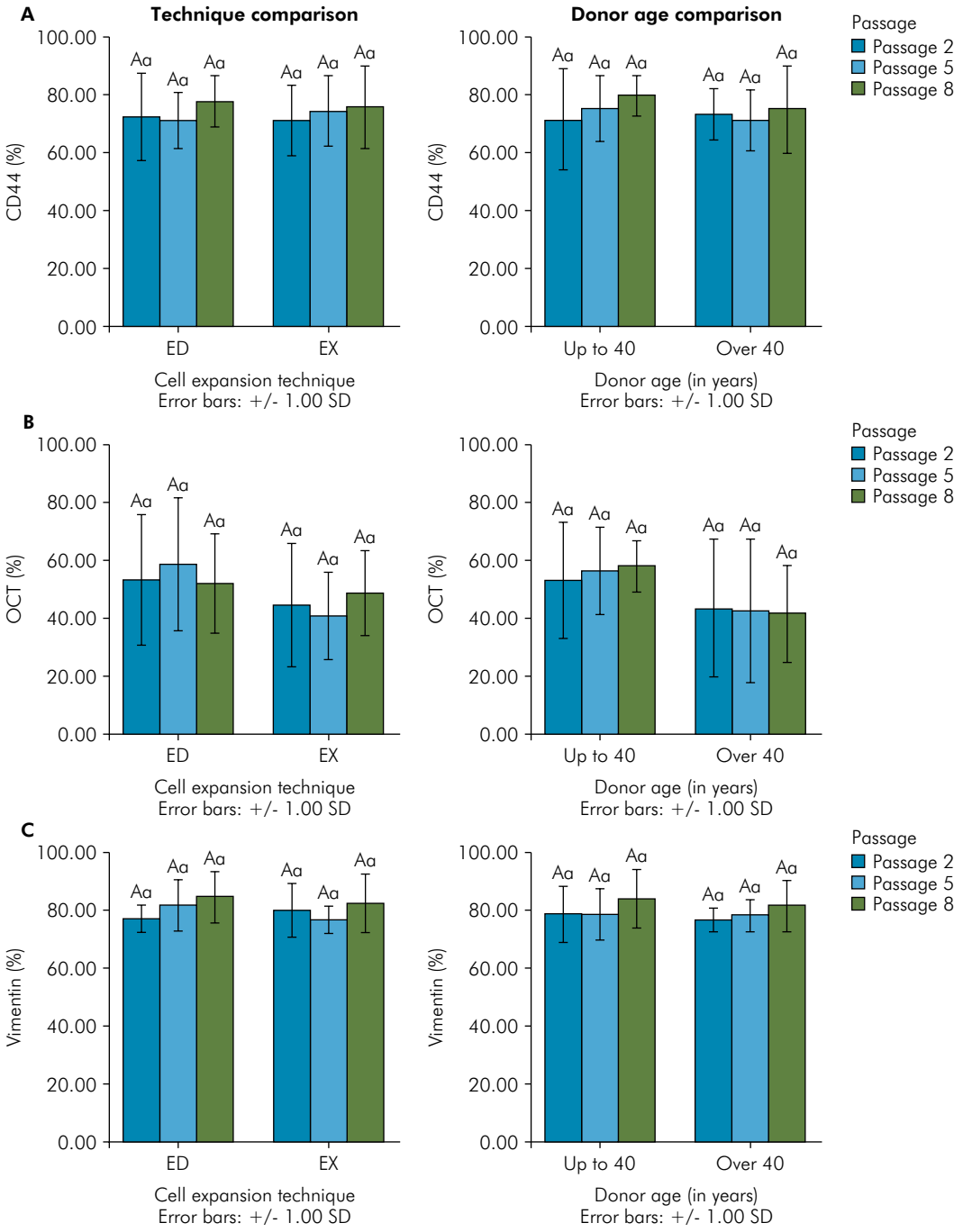

Figure 5. Concentration (%) of dental pulp stem cells (DPSCs) positively marked with CD44 (A), OCT (B) and vimentin (C) in passages 2, 5 and 8.In the left column, cell expansion techniques are being compared, while in the right column, the comparison is between donor age (in years) groups (up to 40/over 40). There was no significant statistical difference detected for all comparisons (ANOVA, $p > 0.05$).

Immunofluorescence

Figure 7 shows the labeling of DPSCs for CD44, nestin, OCT, vimentin and CD146. All markers presented high labeling according to fluorescence microscope images (×20).

Discussion

In the present study, it was observed that while there was no difference between the expansion

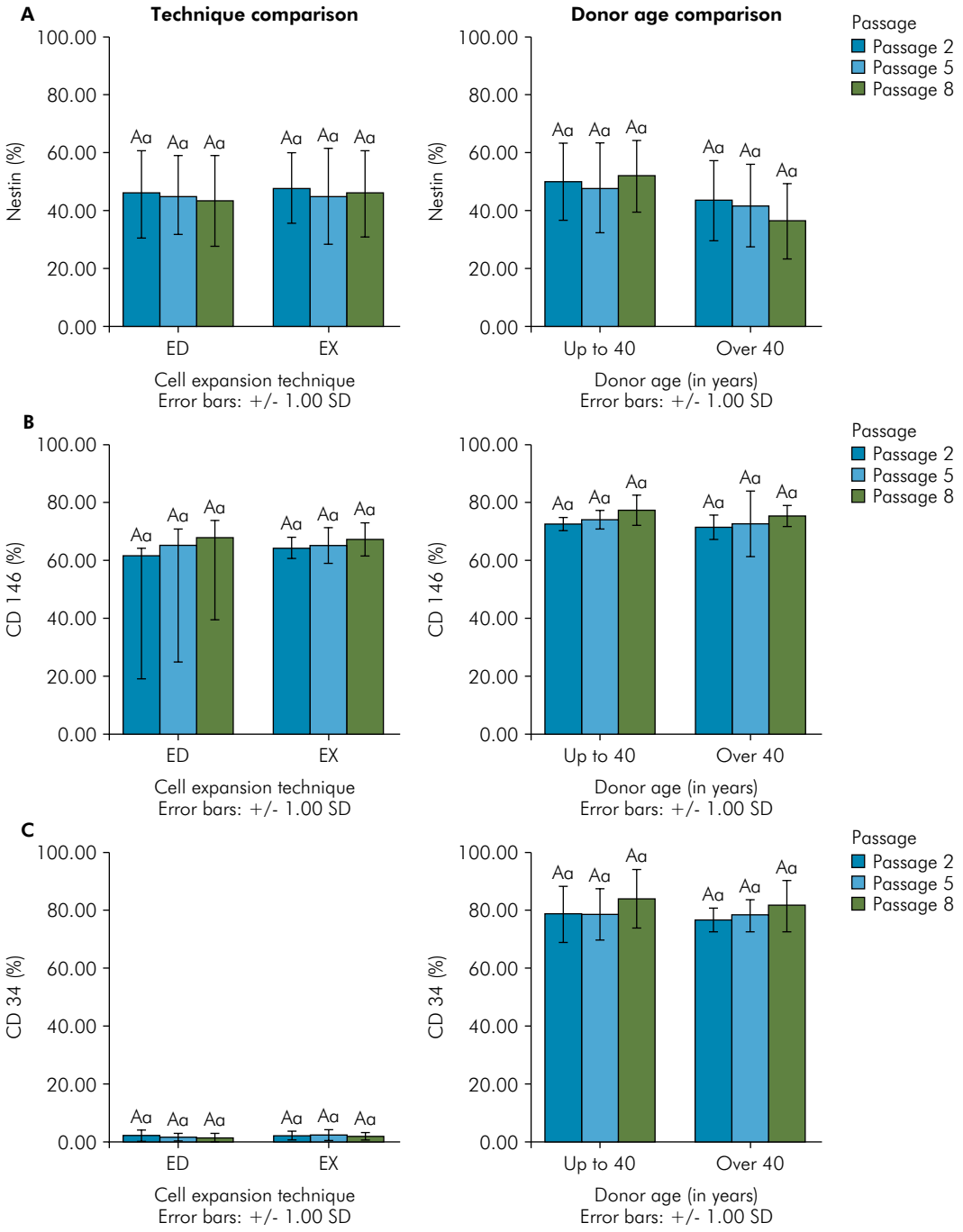

Figure 6. Concentration (%) of dental pulp stem cells (DPSCs) positively marked with nestin (A), CD146 (B) and CD34 (C) in passages 2, 5 and 8. In the left column, cell expansion techniques are being compared, while in the right column, the comparison is between donor age (in years) groups (up to 40/over 40). There was no significant statistical difference detected for all comparisons (ANOVA, $p>0.05$).

technique, passage or age, the best optimization values were found using the ED technique for individuals up to 40 years old. It is important to define the optimum conditions considering the isolation and applications of dental pulp stem cells (DPSCs) in different therapies because factors such as donor age, number of passages and techniques may interfere with cell quality and stemness. Some studies have

Table. Maximum desirability values for each marker (CD44, OCT, nestin, vimentin and CD146) and D values considering all markers for each situation (ED/up to 40, ED/over 40, EX/up to 40 and EX/over 40).

Marker	Maximum D value (ED/up to 40)	Maximum D value (ED/over 40)	Maximum D value (EX/up to 40)	Maximum D value (EX/over 40)
CD44	0.97620	0.97666	0.98600	0.97620
OCT	0.87297	0.63326	0.80328	0.87297
Nestin	0.83414	0.76181	0.89603	0.83413
Vimentin	1.00000	0.99817	0.99847	1.00000
CD146	0.97411	0.98097	0.98927	0.97411
D value	0.92475	0.63326	0.90427	0.90316

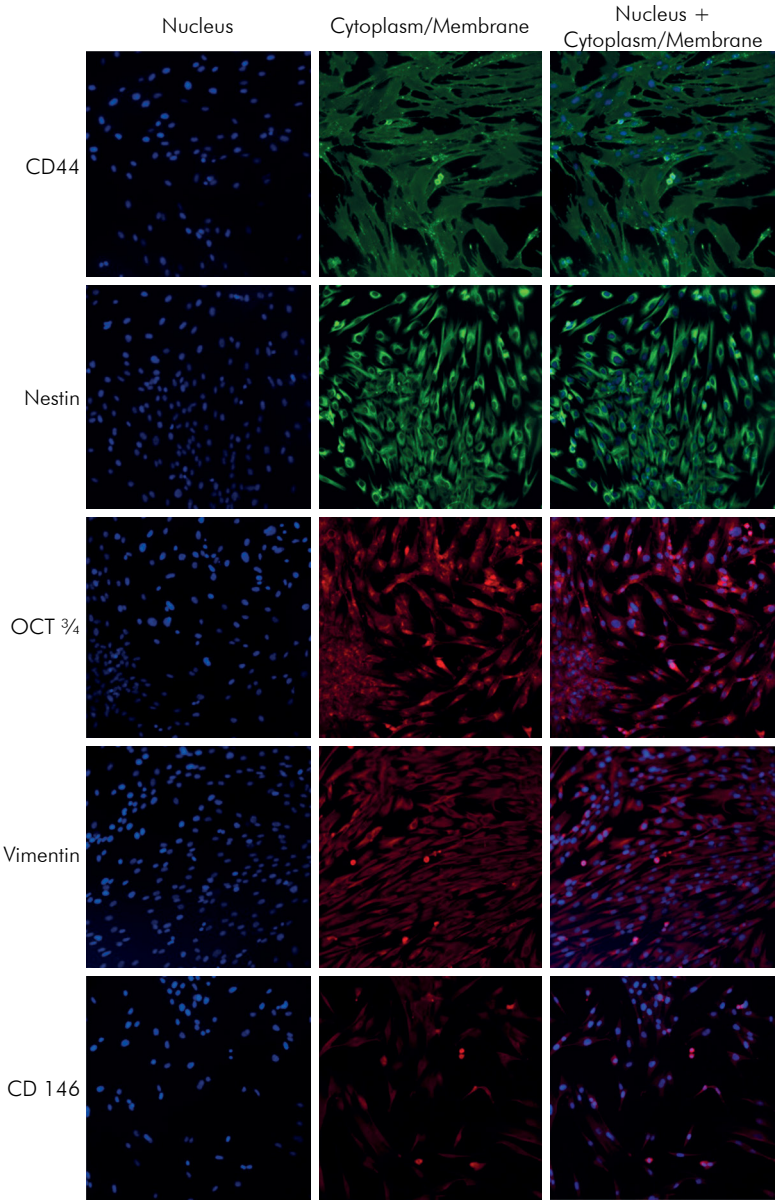

Figure 7. Panel representing the microscope fluorescence images of dental pulp stem cells (DPSCs, EX technique, passage 5, 29-year-old donor) marked positively for CD44, CD146 (cell membrane) Vimentin, Nestin and OCT3/4 (cytoplasm). X20.

compared each of these conditions in an isolated manner[27-29]. However, the optimization of the best condition, considering the association of all three factors, has not yet been described.

It was observed that regardless of the donor age, passage or technique, a high concentration of vimentin (80%) and CD44 (75%) and an intermediary concentration of nestin (45%), OCT (50%) and CD146 (65%) were found, whereas CD34 presented a negative labeling as expected (2%). Moreover, the inverted microscope and immunofluorescence images indicated a lack of morphological, labeling and confluence differences among groups when the conditions were changed. These results are important for guiding future clinical research that will use stem cells in the treatment of different types of diseases.

Regarding the comparison between enzymatic digestion and explant, Gopinath et al.[30] observed that enzymatic digestion had higher osteo/odontogenic differentiation potential compared to explant technique from young patients with inflamed dental pulp tissues. Conversely, Jeon et al.[28] found no significant difference for CD90 and CD105, morphologic characteristics, proliferation and osteogenic differentiation, whereas ED was superior thanEX for colony-forming units, Stro-1, CD146 and adipogenic differentiation. It is important to highlight that Jeon et al.[28] analyzed only SHEDs and used different pulp tissues for enzymatic disaggregation and explant protocols.

In our study, samples from the same pulp tissue were used to compare the morphology, proliferation capacity during cell expansion and stem cell markers for both techniques. That might explain the lack of difference found for those response factors. Likewise, Hilkens et al.[27] compared the two techniques in permanent teeth using the same pulp tissue and found no significant difference regarding the proliferation rate, colony formation, mesenchymal stem cell markers and multilineage differentiation potential. However, the authors only analyzed early passages (2–4). It was shown that, even for late passages (8), both techniques are similar with respect to the variables investigated in this study.

The number of passages is another factor that can interfere with cell functions. Martin Piedra et al.[31] analyzed late passages of DPSCs considering cell viability levels, apoptosis and cell proliferation. The authors found high average cell viability and proliferation levels for late passages (11–-14) and the activation of preapoptotic processes from passages 15–20. Although this is an important information when working with DPSCs, the authors did not investigate stem cell markers in those late passages.

Similar concentrations of mesenchymal cell markers were found for passages 2, 5 and 8, although the number of days necessary to reach confluence was slightly higher for passage 8. Lizier et al.[22] evaluated the influence of passages for SHEDs and found that the number of cells increased in passages 4–8, and after passage 9 this number decreased. Additionally, apoptotic activation can occur after the 10th passage. It is shown that up to passage 8, cells can present high levels of stem cell markers and a good proliferation rate,[22] but further studies are necessary to evaluate late passages to define a cut off point for tissue regeneration.

Regarding donor age, there are few studies comparing young and old donors. Bressan et al.[25] compared adult donors aged 16 to > 66 years and showed that all donors presented a good proliferative ability at passage 2, and the osteogenic differentiation ability was the same for all donors. Ning et al.[32] observed that in an inflammatory microenvironment, DPSCs from adult rats presented weaker repair capacity than younger donors due to damage noticed in proliferation and mineralization rates. These results complement our findings that indicate no difference in stem cell markers for different ages.

Although the oldest donor showed the worst proliferation capacity at passage 8, the difference was not discrepant when compared with that of the other donors. In addition, no significant differences were found regarding to the stem cells markers evaluated, then the immunophenotyping of all groups were similar regardless of age. This study evaluated a small number of donors, which is a limiting factor, and more studies with a higher number of individuals are necessary to confirm the lack of difference among donor ages. Other limitation of this study was the number of pulp cells obtained to perform many different tests, once it was a priority to evaluate de natural expansion of the pulp cells (from the tissue obtaining until passage 8 expansion), without freeze

the cells. Future assays like MTT, Live/Dead and the immunophenotyping using other stem cells markers are suggested.

As discussed, despite the important findings related to stem cells from pulp tissue, many investigations are needed to allow the adequate use of DPSCs on regenerative tissue in humans. Some studies investigating important issues such as cryopreservation, the use of scaffolds in association with cells and cell behavior in tissues have presented promising data[33,34] however, many questions need to be better clarified. The results from the present study help to understand the influence of different factors regarding to the cell proliferation rate and cell phenotype during their natural growth. It was concluded that neither the isolation technique nor the donor age or cell passage significantly interfered with the stem cell phenotype and proliferation rate during natural cell expansion.

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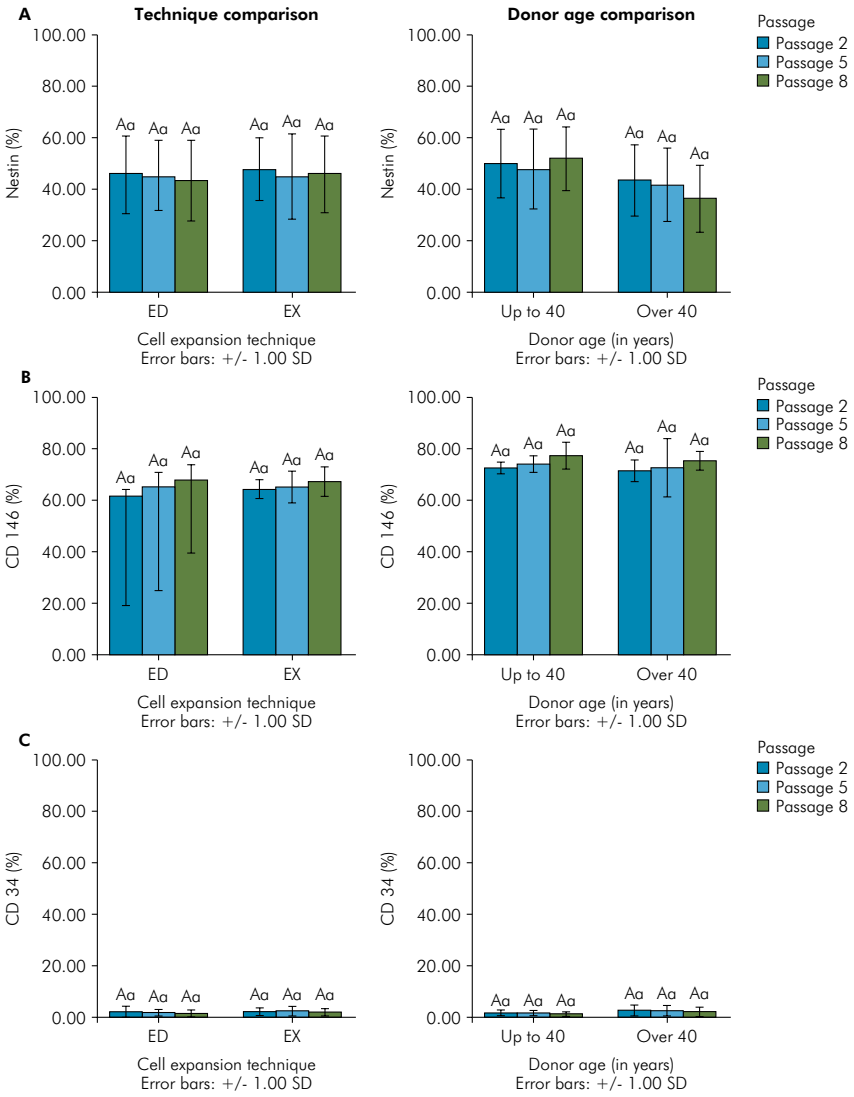
Proliferation rate and expression of stem cells markers during expansion in primary culture of pulp cells. Braz Oral Res. 2021;35:e128.

Figure 6.
Part C has been replaced with the correct graphics.



https://doi.org/10.1590/1807-3107bor-2021.vol35.0128.erratum