Brazilian Dental Science
Ciência Odontológica Brasileira
unesp
UNIVERSIDADE ESTADUAL PAULISTA "JÚLIO DE MESQUITA FILHO"
Instituto de Ciência e Tecnologia
Campus de São José dos Campos

ORIGINAL ARTICLE

doi: 10.14295/bds.2020.v23i1.1753

Isolation and Characterization of Stem Cells Derived by Human Dental Pulp from Harvest Based in Rotary and Manual Techniques used in Endodontic Therapy

Isolamento e caracterização de células tronco da polpa dentária humana obtidas em instrumentação mecanizada e manual durante tratamento endodôntico.

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ABSTRACT

Objective: The aim of this study was to evaluate the impact on the isolation and characterization of stem cells from pulp tissues obtained through rotary instrumentation techniques compared to the manual technique. **Material and Methods:** Thirty permanent teeth were included, 15 of which were instrumented with rotational technique (Protaper SX) and other 15 with manual technique. Cells obtained were characterized by flow cytometry and proliferation was evaluated by the MTT assay. The plasticity was evaluated for adipogenic, osteogenic and odontogenic differentiations. **Results:** Cells isolated from the pulp of permanent teeth, by manual techniques, presented fibroblast morphology and were able to differentiate successfully. All lineages expressed CD29, CD73, CD90, CD105, CD146, CD166 and were negative for CD31, CD34 and CD45. MTT assay showing significantly increased proliferation of hDPSCs in 5 and 7 days of the culture. **Conclusions:** The present study demonstrated that manual instrumentation technique is one of the best candidates to harvest dental pulp tissue as the dental stem cell source due to ability effective expanded with less tissue invasion. The technique of rotational instrumentation proved to be very harmful to the tissues of the dental pulp, and we can't obtain cells using this technique.

KEYWORDS

Root Canal Therapy; Pulpectomy; Anatomy and Histology; Stem Cell.

RESUMO

Objetivo: O objetivo deste estudo foi avaliar o impacto no isolamento e caracterização de células-tronco de tecidos pulpares obtidos por meio de técnicas de instrumentação rotatória em comparação à técnica manual. **Material e Métodos:** Trinta dentes permanentes foram incluídos, 15 dos quais foram instrumentados com técnica mecanizada (Protaper SX) e outros 15 com técnica manual. As células obtidas foram caracterizadas por citometria de fluxo e a proliferação foi avaliada pelo ensaio MTT. A plasticidade foi avaliada quanto às diferenciações adipogênica, osteogênica e odontogênica. **Resultados:** células isoladas da polpa de dentes permanentes, por técnicas manuais, apresentaram morfologia de fibroblastos e foram capazes de se diferenciar com sucesso. Todas as linhagens expressaram CD29, CD73, CD90, CD105, CD146, CD166 e foram negativas para CD31, CD34 e CD45. O teste de MTT mostrou proliferação significativamente aumentada de hDPSCs em 5 e 7 dias da cultura. **Conclusões:** O presente estudo demonstrou que a técnica de instrumentação manual é um dos melhores candidatos para a colheita de tecido pulpar como fonte de células tronco dentárias devido à boa capacidade de proliferação celular com menor invasão tecidual. A técnica de instrumentação rotatória provou ser muito prejudicial para os tecidos da polpa dentária, e não possibilitou obter células.

PALAVRAS-CHAVE

Anatomia e Histologia; Célula tronco; Endodontia; Pulpectomia.

INTRODUCTION

In recent years, mesenchymal stem cells (MSCs) derived from dental tissues have been studied because of their ease of achievement and potential cell therapy use [1-4]. They are also generally homogenous and proliferate more rapidly than bone marrow mesenchymal stem cells (BMSCs) and many other types of MSCs [5]. Human dental pulp stem cells (hDPSCs) were isolated for the first time in the year 2000 [1]. Previous research on hDPSCs has been based primarily on pulpal tissues derived from healthy primary incisors and permanent teeth from Third molars. The pulp contains a heterogeneous population of cells, such as fibroblasts, inflammatory cells, nerves, vessels, and perivascular cells [2, 3]. These stem cells are also relatively easy to collect from naturally lost, surgically removed teeth [6,7] or in a pulpectomy procedure indicated in a irreversible pulpitis and also in cases of healthy pulp (indicated for support of prosthesis, for example)[8, 9]. Pulp removal can be performed with manual or mechanized instrumentation; however, according to the literature, there are few reports on mechanized instrumentation in obtaining stem cells. Therefore, the objective of the present study was thus, the objective of the present study was to evaluate the efficiency of obtaining stem cells by two techniques.

MATERIAL AND METHODS

To confirm the quality pattern of DPSC obtained, we selected two samples cultured with success from the manual instrumentation group. A donor of the pulp was 18 years of age and the other sample was from a donor with 60 years (called P68 samples and P73 respectively). Differentiation assay; cell viability analysis by MTT assay and flow cytometric was performed. Histological evaluation of human dental pulp was performed both manual and mechanized instrumentation groups.

ISOLATION AND CULTURE OF HDPSCS

This study was approved by the Research Ethics Committee on Human Subjects (CAEE 43823115600005133), and all patients were followed up as a post-treatment endodontic protocol, without prejudice to the patient, regardless of inclusion in the study. All vital of permanent teeth of patients over the age of 18-years, was removed with a clinical indication for the procedure was included in the survey. Fifteen patients that had two pulpectomies simultaneously, they had one removal by manual technique and the other by mechanized technique. Teeth with fistulas, edema, dark crowns, mobility or without vitality were excluded, as well as elements that did not allow to use absolute isolation for the removal of the pulp... The tissue removed was maintained in a sterile tube containing a balanced saline solution (1X PBS) until manipulated.. In the primary culture was performed, and the pulp was fragmented with a scalpel and in 60 mm Petri dishes with Eagle's medium (α-MEM) supplemented with 10% FBS, 2 mM L-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin and then incubated at 37 °C in 5% CO2. Medium was changed each tree days. [10, 11]

Differentiation of hDPSCs

Adipogenic differentiation

Cells (P4) were seeded onto 60-mm culture dishes (400 cells/dish) and cultured for 12 days in the MSC-growth medium. Cells were then subcultured for 21 days [12] in adipogenic induction medium comprising α-MEM supplemented with 5% FBS, 10−6 M dexamethasone (Sigma-Aldrich), 5 μg/mL insulin (Sigma-Aldrich), and 50μM indomethacin (Sigma-Aldrich). After culturing, cells were fixed in 10% neutral buffered formalin for 10 minutes, and then stained with Oil red-O (Sigma-Aldrich) solution for 30 minutes.[11, 13]

Osteogenic differentiation

Osteogenic differentiation, about $5x10^3$ cells (P4) /well were grown in 6 wells plates. After 24 hours, the basal medium was replaced by DMEM insensitive Low Glucose medium (DMEM-LG; Gibco) supplemented with 2% (v/v) of Knockout serum (Gibco), 1% (v/v) Penicillin/Streptomycin and osteogenic inductors (100 μM dexamethasone and 50 μM 2-Ascorbic acid phosphate - Sigma-Aldrich). The trading of medium was held every three or four days. On day 10 of differentiation, both culture media (experimental and control) have been redone being supplemented with 200 mM of β- Glycerophosphate (Sigma). After 21 days of cultivation, the samples were evaluated by Von

Kossa staining to determine mineral deposition. [11, 12, 14].

Odontogenic differentiation

The cells (P4) were seeded in 6-well plates at a density of $5x10^3$ cells/well to quantify mineralized nodule formation *in vitro*. The cells were incubated in medium supplemented with 10% FBS until they reached subconfluence. The medium was then switched to osteogenic differentiation medium containing 10 nM dexamethasone, 10 mM b-glycerophosphate, 50 mg/mL of ascorbate phosphate (all from Sigma-Aldrich), and 10% FBS. The medium was changed every 2 days. Mineralization was detected and quantified weekly using an Alizarin red-based assay over 21 days of cultivation. Briefly, cells in 6-well plates were washed with PBS and fixed in 4% (v/v) phosphate-buffered paraformaldehyde at room temperature for 60 min. The cells were then washed twice with an excess of dH2O, after which 250 mL of 40 mM Alizarin Red S (Sigma-Aldrich; pH = 4.1) was added to each well. The plates were incubated for 20 min with gentle shaking. After aspirating unincorporated dye, the wells were washed four times with 1 mL of dH2O with 5 min of shaking for each washing step. The stained monolayers were visualized with an inverted microscope. [15-17].

Flow cytometric

The analysis of flow cytometry was carried out on crops from the dental pulp by the method of manual instrumentation on fifth passage. A donor of the pulp was 18 years of age and the other sample was from a donor with 60 years (P68 samples and P73). To evaluate the immunophenotype profile of these cells, the enzymatic breakdown was performed with TrypLeExpress (Thermo Fisher Scientific). Were incubated with conjugated antibodies in sheltered location 4° C light, washed twice with PBS (1x) [18] without calcium and magnesium and then fixed with the cell permeabilization Kit (Invitrogen, GAS004). We used the following antibodies: FITC Mouse Anti Human CD31 (BD Kit 555445), APC Mouse Anti Human CD73 (BD Kit 560847), PE Mouse Anti Human CD90 (BD Kit 555596), PE Mouse Anti Human CD166 (BD Kit 559263), and FITC, PE and APC Mouse IgG1 Isotype Controls κ (BD 555748 Kit, 554681 and 555749, respectively). CD29 (BD Kit: 559883); CD146 (BD Kit 550315); CD105: (BD Kit 561443); CD34: (BD Kit 555824); CD45 (BD Kit 555482) following the manufacturer's recommendations. A minimum of 10,000 events was analyzed in the Guava Easy Cyte (Guava Technologies) and analyzed the results in FlowJo software – Single Cells Analysis.[11, 19] .

Histological evaluation of Human Dental Pulp

The dental pulps were fixed in 10% formalin (Sigma-ALdrich, St. Louis, USA) to hematoxylin and eosin staining and 4% paraformaldehyde (Sigma-Aldrich) in 0, 1 m phosphate buffer pH 7.4 for immunohistochemical technique. Tissue fixation processes were made for 72 hours at 4ºc. From this, the samples were washed in running water for 10 minutes and the dehydration was made using immersions of pulps in increasing ethanol concentrations (70%, 80%, 90%) and twice 100% ethanol for 1 hour each. After the polymerisation of paraffin, the blocks containing the samples were cut into automatic microtome RM2165 Leica (Leica, Wetzear, Germany) producing sequential cuts with approximately 5 μm. The cuts were carefully seated on slides histological and left in an oven at 60° C for 16 hours for a better fixation of samples and subsequent evaporation of residual paraffin. For the realization of the staining by hematoxylin-eosin, the dental tissue sections were dew axed with two dips in xylene, followed by subsequent dips in ethanol concentrations (100%, 70%, 95% for 10 minutes each and finally, immersion in water 5minutes distilled. Then, the blades were placed on Harris Hematoxylin staining (Sigma-Aldrich) for 1 minute and then in water for 10 minutes, to remove the excess dye. The slides were then stained in Eosin (Sigma-Aldrich) for 3 minutes later the dehydration process with 95% ethanol and two dips in 100% ethanol for 5 minutes each. The slides were mounted with Permount ® (Fischer Scientific, Pittsburg, USA).

Cell viability analysis by MTT assay

To assess the pattern of proliferation of isolated stem cells, was used the test of MTT (3-bromide [4.5-dimethyl-thiazole ring-2-yl]-2.5-diphenyltetrazolium, Sigma). In the essay, 500 cells/well were initially seeded (day 0) in a 96-well plate (Corning) and the readings were held in 3,5 and 7 days. The medium was removed and added 180 μL of pure medium plus

20 μL of MTT. Then the board was kept in the dark in the oven for 4 hours, and to assess the pattern of proliferation of isolated stem cells, was used the test of MTT. In the essay, 500 cells/ well were initially seeded (day 0) in a 96-well plate (Corning). The medium was removed and added 180 μ l of pure medium plus 20 μL of MTT. Then the board was kept in the dark in the oven for 4 hours and, after this period, the MTT was removed and 200μL of isopropyl alcohol were added. The board was kept in the dark in the oven for 1 hour; the supernatant was transferred to a new card and like 200 μL control of acidified isopropyl alcohol were added. The proliferation was evaluated in the spectrophotometer absorbance of 570nm [9, 11].

Statistical analysis

The cellular metabolism dates were tested by one-way analysis of variance (ANOVA). The test statistical significance was determined at $p < 0.05$. The experiment was performed three times.

RESULTS

Culturing the isolated cells

Fifteen patients had samples collected both by rotary instrumentation and by manual instrumentation. With the employed methodology, it was possible to obtain stem cells by the manual instrumentation method. The rotary instrumentation method did not obtain any stem cell samples. The totals of samples collected were grouped in the following table (Table 1), as well as the respective mean age and contamination observed in each group.

Fibroblast-like adherent cell cultures were established from manual instrumentation (Figure 1). Thus, these results showed that dental pulp removed from manual instrumentation contains viable cells. Furthermore, no cells were detected using rotational instrumentation, it is observed that the haversted pulp is already highly fragmented, leaving the material semi-crushed (Figure 1).

Table 1 - Summary of variables treated per group of treatment.

Groups of tratment	Sample Size	Cells cultured	Sample Contami-nated	Aged, Median (range)
Rotary technique	15	0 (0%)	2 (14%)	32.4 years
Manual technique	15	8 (53.4%)	1 (7%)	35.7 years

Differentiation potential of hDPSCs

Only cells in the samples taken were obtained by the method of manual instrumentation. As a result, we realized that the cells were able to differentiate into osteogenic, adipogenic and odontogenic lineages, in a manner compatible with the typical HDPSC plasticity (Figure 2).

Analysis by MTT assay

The MTT test conducted with strains allowed us to assess the cells obtained are healthy and proliferate within an appropriate standard, keeping your viability throughout the analysis period of cultivation which was 7 days. No difference was observed between 3 and 5 days, but between 5 and 7 days, there was a sharp increase of absorbance, compatible with the increase in population expected to observe after seven days of cultivation [19] (Figure 3).

Flow cytometric analysis

In the immunophenotypic evaluation by flow cytometry, all the samples tested showed expression of: CD29 (P68 99.22%, P73 97.33%), CD90 (P68 99.81%, P73 99.64%), CD166 (P68 91.5%, P73 75.1%, CD146 (P68 34.4%, P73 19.43%), CD73 (P68 99.08%, P73 100%), CD105 (P68 85%, P73 39%). CD34 (P68 1.85%, P73 1.28%) CD34 (P68 0.5%, P73 0.4%) CD45 (P68 0.91%, P73 1.32%) was also found to be lacking expression of the hematopoietic cell marker CD31 (P68 1.85%, P73 1.28%). These data were compatible with the results obtained in the literature review [20]with respect to the immunophenotypic characterization of MSCs (Figure 4).

Histological evaluation

To further elucidate the nature of the material obtained in the two groups, an additional study was carried out for the histological analysis of the obtained material. The dental pulps were obtained under the same conditions described above. Areas were observed suggesting trauma or temperature necrosis in the sample collection process. In a sample obtained by rotational instrumentation, alternating areas of tissue were observed, generating a pattern compatible with the very design of the endodontic instrument used during the removal of these pulps (NiTi ProTaper® SX). These areas demonstrated a degradation of collagen compatible with that caused by denaturation due to heating (Figure 5).

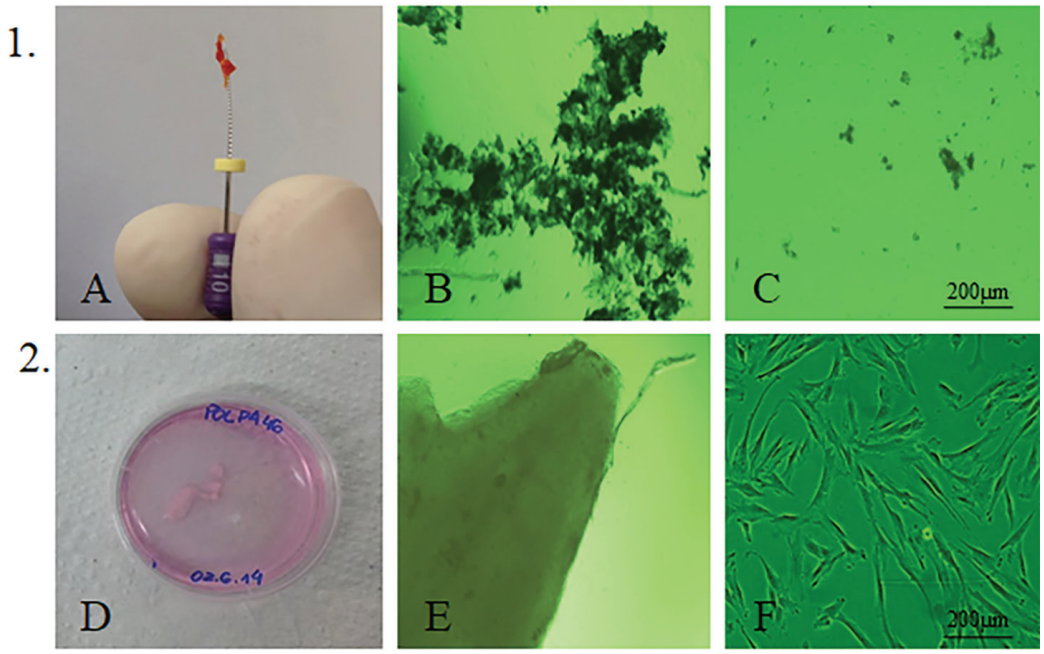


Figure 1 - Isolation and culturing of hDPSCs obtained from both the endodontic techniques: 1. (A, Freshly isolated pulp tissue by manual instrumentation; B, dental pulp in culture and (C) no presence of cells on day 7 at passage 0 (x 40); 2. (D, dental pulp harvested by manual instrument; B, dental pulp in culture and (C) Higher magnification of the cells on day 7 at passage 0 showing the fibroblast like satellites shape of the cells. (x 100). Scale bar, 200 µm.

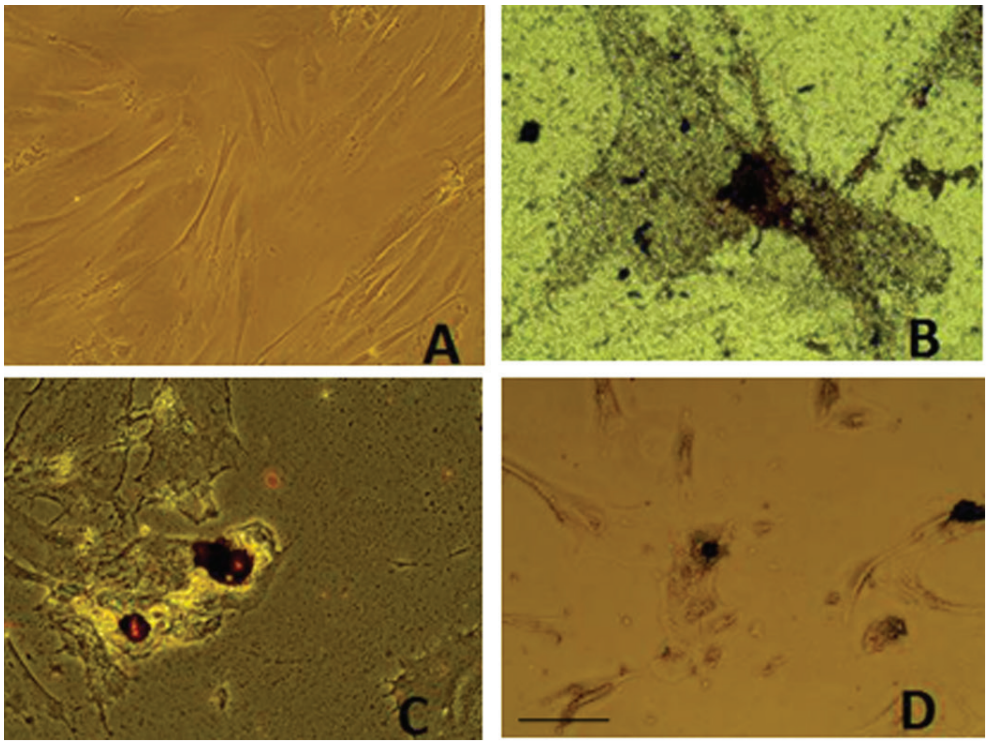
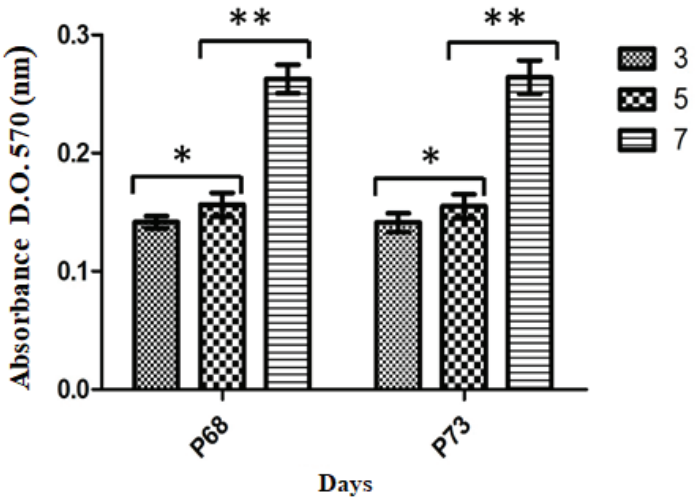


Figure 2 - Differentiation potential of dental pulp cells. (A) Undifferentiated hDPSCs - negative control (x 100). (B) Osteogenic differentiation potential of pulp cells evidenced by mineralized matrix deposition stained with Alizarin Red stain. (x 200). (C) Adipogenic differentiation of human dental pulp stem cells (hDPSCs) stained with Oil Red stain, evidenced by lipid droplets stained (x 200). (D) Odontogenic differentiation potential of pulp cells stained with Alizarin Red stain (x 100). Scale bar, 200 µm.



* P > 0.05 between 3 and 5 days.
** P < 0.05 (0.001) between 5 and 7 days or 3 and 7 days.

Figure 3 - MTT assay showing significantly enhanced proliferation of in hDPSC compared between 5 and 7 days or 3 and 7 days.

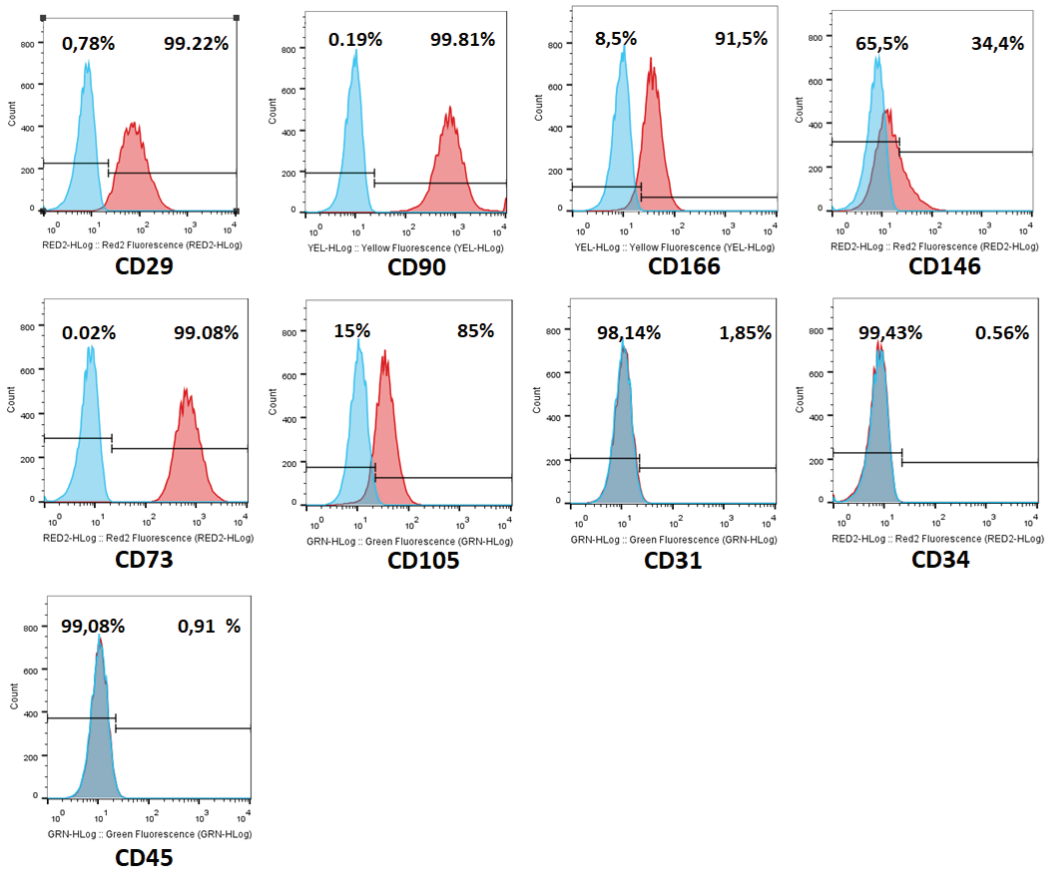


Figure 4 - The human hDPSCs from manual technique showed high expression for surface markers CD29, CD73, CD90, CD105, and CD166; a median expression for CD146 but very low expression for CD31, CD34, and CD45 (blue line, isotype control; red line, marker of interest; Max, maximum).

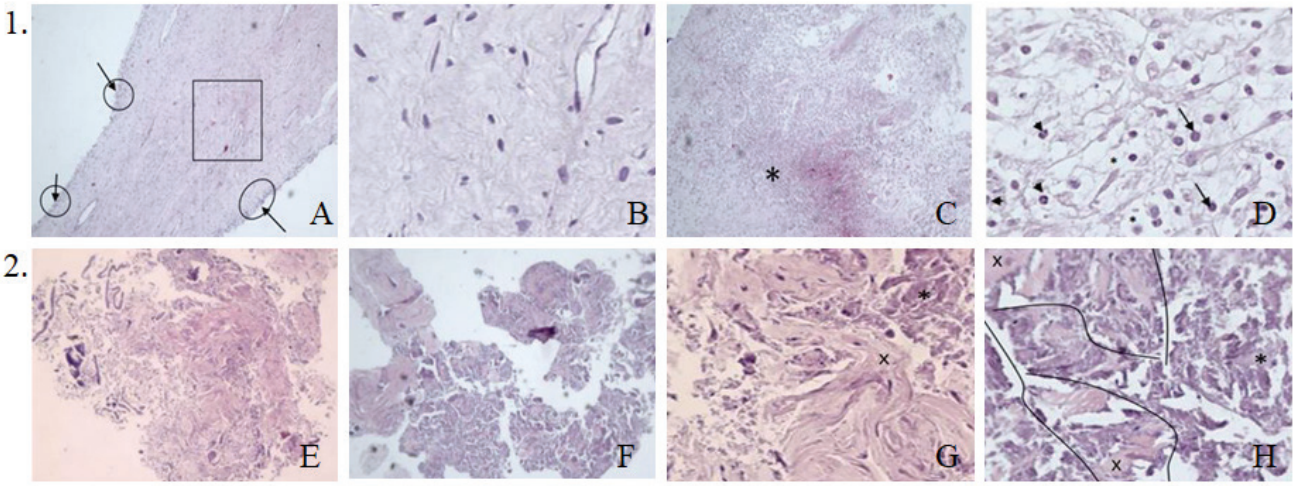


Figure 5 - Images from histological preparation: 1. Manual instrumentation, hematoxylin and eosin (H&E) staining. (Panoramic 200X); (Details 400X). 2. Rotary instrumentation, hematoxylin and eosin H&E staining. (Panoramic 200X); (Details 400X). 1.A. It showed the native structures of pulp were well-preserved, the preservation of the vascular and nervous architecture, as well as the cellularity in the central region (area delimited by the square); 1.B. Preservation of the subodontoblast and, possibly, odontoblastic layers (showed on Figure 1.A.: circles and arrows); 1.C and 1.D. It exhibited moderate polymorphonuclear (arrow head) and mononuclear (arrow) mixed inflammatory reaction and diffuse inflammatory reaction associated with edema (asterisk). 2. E and 2.F. It showed pattern areas in structures of pulp suggestive of necrosis and tissue disorganization with tissue loss integrity, suggesting trauma or temperature necrosis in the process for obtaining the sample. 2. G and 2.H. Basophilic regions suggestive of pulpal calcification (asterisk), as well as the eosinophilic areas (X), in these regions no preservation of vascular, nerve structures or possible cellular characterization of structures of pulp.

DISCUSSION

The investigation for new sources of mesenchymal stem cells (MSC) as an alternative to stem cells derived from bone marrow (BMSC) has been increasing in the last decade mainly in human medicine. In this way, the dental pulp stands out as a source of easy collection, non-invasive harvest performed during endodontic treatment and with a great possibility of dental banking storage making it applicable for cell therapy in human medicine. In the present study, a randomized trial that compared manual and rotary techniques of extraction pulp tissues of third molar teeth instrumentation to obtain human dental pulp stem cell (hDPSC). Studies have compared rotary with manual instrumentation performance for root canal preparation in permanent teeth [3,21]. In these researches, the results have shown no significant differences in the cleaning capacity between techniques, but rotary instrumentation prepared canals more rapidly and uniformly [21]. In addition, the rotary instruments can reach the entire length of the root canal, causing little or no displacement, particularly in the apical region [21]. However, in this paper, the nature of the material obtained was highly different in rotary instrumentation compared from the material removed manually. Variations in the form of pulp removal can change the final characteristic of the material removed. When removed manually, the pulp is obtained in larger and more structured parts. In the mechanized form, a macerated material is obtained, semi-crushed. Thus, it was not possible to compare samples between the groups, considering that no cells were obtained of dental pulp harvested samples from rotary instrumentation group. Dental pulp stem cells from permanent teeth only were isolated in the manual instrumentation group. Despite the failure in rotary instrumentation purpose, the pulp removed by this method is supported as a proposal, based on laboratory questions. However, using rotary instrumentation, it is observed that the pulp obtained is already highly fragmented, leaving the material semi-crushed. This would theoretically be an advantage of this method. In previous studies [9,22] the dental

pulp retrieved from the tooth was mechanically dissected into small pieces (tissue explants) and that these parts are better exposed to the culture medium during initial dental pulp outgrowth culture. Although was also observed the success rate in establishing cell culture from inflamed dental pulps was lower than that verified for normal teeth [23].This was probably due to the fact that the amount of tissue obtained from inflamed teeth is usually lower. Other paper showed that cells from inflamed pulps presented a late emergence of single-cell-derived colonies. [24] This fact is probably associated with a lower volume of tissue one normally obtains following pulpectomy of inflamed teeth, rather than to a compromised cell phenotype.

According previous studies [25], using instrumentation over root canals the external radicular temperature can raise he thermal variation between 0.4ºC ± 1.0ºC. Temperature alterations transmitted to the root surface may be harmful to the surrounding tissues, because the heat generated inside the canal dissipates through dentine, reaching the cementum, periodontal ligament and alveolar bone [25]. Some histological aspects may explain the data presented in this work and corroborate the findings of others reports [25, 26]. In this study, the evaluation of dental pulp conditions (after harvest by rotary and manual techniques) was evaluated by histological analyses, which do not consider immunohistochemistry features. Areas were observed suggesting trauma or temperature necrosis in the rotary group samples collection process. These areas demonstrated a collagen degradation it was compatible with denaturation caused due to heating. These results suggest that this increase in temperature may be the factor that impeded success in the rotary instrumentation group in isolation stem cells, whereas this temperature raise did not occur in the manual instrumentation group. In the manual instrumentation group structures of pulp were well-preserved, the preservation of the vascular and nervous architecture, as well as the cellularity in the central region. Despite this, studies that showed the clinical use of rotary nickel-titanium (NiTi) endodontic instruments improves the efficiency of endodontic treatment by reducing the time spent on treatment procedures, sensitivity and complication risks [26].

In this paper, it has further shown the feasibility of deriving hDPSCs form manual instrumentations technical. In addition, it has shown that these hDPSCs possess the adipogenic, osteogenic and odontogenic differentiation capacity as well as show the stem cell markers. Thus, these results demonstrate that hDPSCs possess stem-cell-like qualities, including self-renewal capability and multi-lineage differentiation and their expression profiles are similar to those of bone marrow stromal stem cells (BMSCs) [27]. These findings may enhance the development of a novel biologically based new generation of clinical materials for regenerative dental and medical treatments [8]The phenotype presented by hDPSCs was compatible to mesenchymal stem cells all fulfilling the criteria from international society of cell therapy for MSC definition [28, 29] and expression of the surface antigens [30] CD29, CD73, CD90, CD105, CD146, CD166, as well as by the lack of expression of CD31, CD34 and CD45 surface molecules. The metabolic activity levels, as measured by MTT, to evaluate obtained cells are healthy and proliferate within a suitable pattern, maintaining their viability throughout the 7 day culture period. No statistical differences between the samples from manual instrumentation group were observed through the time course proliferation assay at 3 and 5 days. Nevertheless, the significant increase in absorbance at 5 and 7 days, due to cells expansion population expected to be observed after 7 days of culture cells.

CONCLUSION

This work describes the successful isolation and characterization of hDPSCs from the pulp tissue by manual instrument technique. Beside, mechanized instrumentation fails to achieve DPSCs. Thus, manual instrumentation is feasible and an important tool to be used in the

endodontic preparation of teeth and can provides to adult patients the chance to also stock dental stem cells without removal of the dental element. Therefore, the pulp might represent a valuable source of hDPSCs, thus making them suitable for dental banking and future therapeutic purposes.

Acknowledgement

Supported by grants of the FAPEMIG, a research supporting foundation of Minas Gerais state. This study was approved by the Institutional Review Board and by the Human Being Research Ethics Committee of the Federal University of Juiz de Fora - UFJF – CEP (in april of 2015, CAAE 43823115.6.0000.513) and is in accordance with the Free and Informed Consent Term (TCLE) used for Experimental and Other Scientific Purposes.

The authors gratefully acknowledge the contributions of Dr Beatriz Julião Vieira Aarestrup from Laboratory of Immunopathology and Experimental Pathology, Biology and Reproduction Centre, Federal University of Juiz de Fora by histological analysis and Dr Luiz Caires from Stem Cells Laboratory of Human Genome and Stem Cells Research Centre, Biosciences Institute, University of Sao Paulo by flow cytometric analysis.

Conflicts of interest

There are no conflicts of interest.

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Date submitted: 2019 Mar 17

Accept submission: 2019 Jul 22