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A N I N T E R N A T I O N A L J O U R N A L

Rat dental pulp stem cells: isolation and phenotypic characterization method aiming bone tissue bioengineering

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ABSTRACT

Dental pulp stem cells (DPSC) have been showing a considerable potential for regenerative medicine. Pulps were collected from lower incisors (n=2) through direct access of the tooth pulp chamber. The isolated cells were cultured in alfa-MEM 10% FBS, in standard culture conditions. At the third passage, DPSC were characterized by flow cytometry (MHCI, CD54, CD73, CD90, CD45, CD11 and CD34); RT-PCR for Nanog gene; and their differentiation capacity in osteogenic, adipogenic and chondrogenic cell lines. Isolated cells exhibited adhesion capacity to plastic; fusiform morphology, and 80% confluence reached in approximately 3 days. These cells have also revealed positive expression for CD54, CD73 and CD90 markers; and negative expression for CD11, CD34 and CD45. Nanog expression was detected by RT-PCR, expected for a mesenchymal stem cell profile. DPSC chondrogenic differentiation was confirmed by positive staining in Alcian Blue; lipidic droplets stained with oil red confirmed their capacity to differentiate in adipogenic fate; while mineralized beads, stained with alizarin red, confirmed their differentiation in osteogenic phenotype. These results indicate the viability of the isolation and expansion of rat DPSC following this method, and osteogenic differentiation potential opens new perspectives for in vivo studies and the use of these cells in cellular therapies and tissue bioengineering, aiming bone repair.

Key words: mesenchymal stem cells, dental pulp, murine, phenotypic characterization, cellular differentiation.

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INTRODUCTION

Cellular therapies and the use of mesenchymal stem cells in tissue bioengineering techniques represent a promise for tissue replacement in various pathologies, including for the treatment of neurodegenerative, cardiac, pulmonary and bone diseases (Iyer et al. 2009; Arvidson et al. 2011; Tang et al. 2012; Dawson et al. 2014). Some clinical studies, especially in the context of coronary heart disease, have boosted the credibility, safety and feasibility of stem cells for human use (Orlicet al. 2001; Strauer et al. 2002; Perinet al. 2003; Santos et al. 2004; Motaet al. 2005).

Cellular therapy protocols indicate that selected cells can be administrated by both (1) intravenous infusion (Bydlowski et al. 2009); or (2) after previous culture in matrices or three-dimensional scaffolds, both as undifferentiated or partially induced cell lines, transplanted for *in loco* repair (Savitz et al. 2002; Atari et al. 2011; Atari et al. 2012). The association of chitosan and embryonic stem cells (Weng-Ning et al. 2009); PGA-PLLA and umbilical cord stem cells (Wu et al. 2004); fibrin scaffold and mesenchymalstem cells (Bensaid et al. 2003); poly (D,L-lactic-*co*-glycolic acid) hydroxyapatite and embryonic stem cells (Kim et al. 2008); phosphate ceramic biphasic calcium and mesenchymal stem cells (Arinzeh et al. 2007); are all examples of stem cells seeded onto biomaterials for tissue reconstruction.

Mesenchymal stem cells (MSC) have been indicated as the first choice in tissue bioengineering studies, by presenting advantages as high differentiation potential, immunosuppressed effects, and viability after expansion in culture (Bensaid et al. 2003; Li et al. 2005; Uematsu et al. 2005; Arinzeh et al. 2007; Lyra et al. 2007; Bertassoli et al. 2013). MSC were originally isolated from bone marrow (Arinzeh et al. 2007). After that, these cells were found in various organs and tissues, including in the dental pulp (Bensaid et al. 2003; Li et al. 2005; Uematsu et al. 2005; Arinzehet al. 2007; Lyra et al. 2007; Bertassoli et al. 2013).

Gronthos and co-workers (2000) published the first report about the isolation and identification of an adult dental pulp progenitor population. Since then, DPSC have been described as a clonogenic cellular population, highly proliferative, capable of self-renew and differentiation in adiponegic, condrogenic, and osteogenic cell lines (Gronthos et al. 2000; Gronthos et al. 2002). Besides the dental pulp from permanent teeth, deciduous and supernumerary teeth have also been used as sources for stem cells (Miura et al. 2003; Kerkiset al. 2006; Jo et al. 2007; Kerkis et al. 2012). However, the stem cells derived from these different teeth differ in the expression pattern of the main markers, and present variations in their differentiation potentials (Jo et al. 2007).

DPSC have been showing positive results for bone repair (Gronthos et al. 2000; Gronthos et al. 2002). These cells can differentiate in osteoblasts and originate bone tissue both *in vitro* and *in vivo* (Lainoet al. 2005, 2006; D'Aquino et al. 2007; Graziano et al. 2008). Some *in vivo* studies have also been showing the potential of the DPSC to improve the ventricular function, to induce angiogenesis and to reduce the infarct size in rats (Gandia et al. 2007); and to differentiate in neural-crest derived melanocytes (Stevens et al. 2008), opening perspectives of their use in regenerative medicine. DPSC also present the classic genetic/antigenic profile, similar to bone marrow mesenchymal stem cells. These cells express both the embryonic stem (ES) cells transcriptional factors, Oct4 and Nanog, as well as MSC surface markers, such as CD105, CD73, and CD13. However, these cells do not express CD45, CD34, CD14, CD43, and HLA-DR (Tang et al. 2012).

An important advantage of the DPSC, compared to other MSC, is that they can be isolated from patients with no major ethical and moral limitations (Prenticeand Tarne, 2007). Additionally, autologous cells do not activate immune rejection, and respond to growth factors inherent to the host. In adults, DPSC isolation is performed after a simple surgical procedure of teeth exodonty, mostly using molars or incisors (Tatullo et al. 2014). DPSC canal so maintain their immnunophenotypic properties and differentiation potential, even after a month of cryopreservation of the whole tooth (Tatullo et al. 2014). Thus, extracted human teeth, usually discarded as biological waste, could become an interesting source of cells for regenerative therapies, aiming not just the development of new teeth, but also other tissues as bones, muscles, and nerves (Telles et al. 2011; Sprio et al. 2012).

Despite their various advantages, the elucidation of DPSC biology is not yet totally clear. Several attempts seek to isolate and characterize adult dental tissue progenitors/stem cells populations, intending to achieve a more defined clonal

population. Understanding the mechanisms involved in the DPSC *in vitro* manipulation and the possible genetic or functional alterations should be considered before using these cells as a therapeutic tool. The present study aimed to isolate and characterize a rat dental pulp stem cell sub-population, collected using a simplified method of isolation. This characterized cell line will be the source for future studies aiming bone tissue bioengineering.

MATERIALS AND METHODS

Animals

Mesenchymal stem cells were collected from dental pulps of lowers incisors of adults Wistar rats (n=2 pulps in each extraction), using animals weighting approximately 250 g. Animals were obtained from the UFMG vivarium (CEBIO). All experiments were performed in accordance with the guidelines established by CEUA/UFMG (Ethics Committee on Animal Use, protocol 288/2013).

Isolation and culture of DPSC

Animals were sacrificed by decapitation after intraperitoneal anaesthesia overdose. After the dissection of the skull, upper and lower jaws were separated and released from the soft tissue. Through a midline incision between the lower incisors, the workpiece was separated into two hemi-jaws. Pieces were immersed in 1% PVP-I and taken to the laminar flow hood for pulp tissue manipulation. With the aid of a scalpel, a small window was opened at the apical foramen, for the pulp tissue exposure (Fig.1A-C). Using a sterile nerve extirpated (size 21 mm, Nº 30 to 40, depending on the foramen diameter), the pulp was released from the root wall, rotating the instrument against the dentinal walls. The pulp tissue was removed without effort or tissue breakdown (Fig. 1C-D).

On a cooled glass plate, the pulp tissue was fragmented and the apical portion, known as apical button (Ohshima et al. 2005) was discarded. The pulp fragments were transferred to a falcon tube for enzymatic digestion with 1,5 mg/mL Collagenase I (Gibco), 40 min at 37°C and 5% CO_2. Digestion was interrupted with growth medium [GM: Minimum Essential Medium Eagle, Alfa Modification (Sigma-Aldric), supplemented with 10% fetal bovine serum (FBS, Gibco), and 1% penicillin/streptomycin/amphotericin B solution (Gibco)]. After centrifugation at 360x g, for 10 minutes at 4°C, the supernatant was discarded and the pellet containing the cells was resuspended in GM. Cells were transferred to 25 cm^2 flasks and incubated at 37°C and 5% CO_2. GM was replaced every 2 to 3 days. After reaching 70% confluence, cells were trypsinized (0.25% Trypsin in EDTA 1mM, Gibco), and transferred to new flasks. Cells were collected for immunophenotypic characterization and differentiation induction at the third passage.

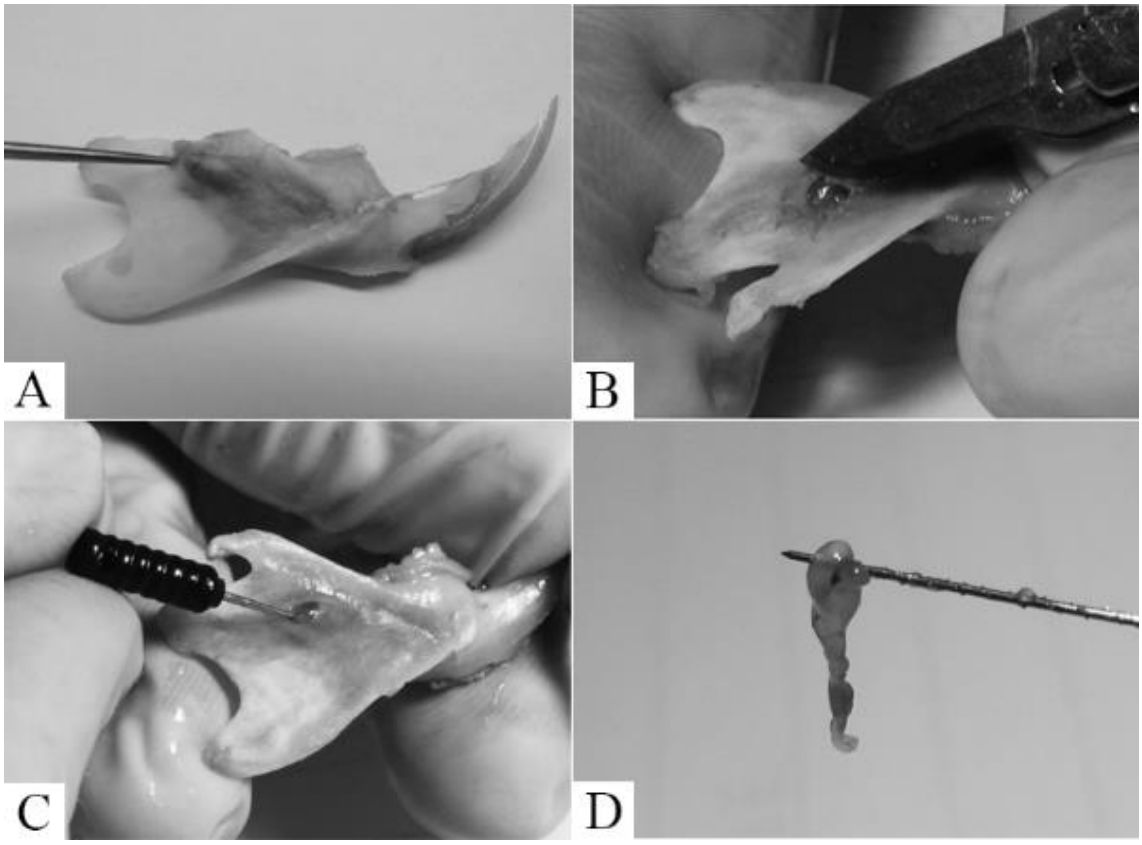

Figure 1. Dental pulp isolation from pulp canal of Wistarrats. In (A), the hemi mandible extracted and dissected. Instrument indicates the end of the apical foramen. In (B), the opening of the apical foramen, used to extract the pulp. (C) Nerve extirpated inside the apical foramen; and in (D), extracted dental pulp.

Flow Cytometry

Antigens typically expressed in MSC (CD54, CD90 and CD73) were used as positive markers; while markers for hematopoietic stem cells (CD45, CD11b/c and CD34) were selected as negative controls. The DPSC immune phenotypic characterization was performed in the third passage. Briefly, $5x10^5$ cells were incubated with monoclonal primary antibody for 30 minutes at 4°C. After washing with PBS, cells were incubated with Alexa Fluor 488 goat anti-mouse (Molecular Probes, Life Tecnologies), diluted 1:500 in PBS, for 30 minutes at 4°C. Background fluorescence was determined in DPSC samples labeled with secondary antibody only. The following antibodies were used: CD11a/b, CD90, CD45, CD54, CD73-phycoerythrin (all from BD Biosciences, USA), CD34 (Santa Cruz Biotechnology, USA), HLA-ABC-FITC (Abcam, USA). Stained cells were next analyzed using the FACS Calibur (BD Biosciences), in 10,000 events, and the data was analyzed using WinMDI 2.8 software. Graphics were generated using data from labeled cells size versus granularity.

Total RNA isolation and reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from the first, second and third passage for the DPSC by using TRIzol Reagent (Thermo Fisher Scientific), according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed in first-strand complementary DNA, following the manufacturer's instructions of the Revert Aid TM H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific). The cDNA was amplified by using *Taq*DNA PolymeraseMaster mix (Thermo Fisher Scientific) and 0.4uM of each specific primer, in a final volume of 25 uL. The sequences of the *Rattus novergicus* Nanog primers were: sense: 5-GGACTGCGGGGACTAAAGG-3, antisense: 5-ACCTGGGGGAGGATAGAGTG-3.

Differentiation potential (chemical induction)

DPSCs were induced to differentiate toward adipogenic, osteogenic, and chondrogenic cell fates for 21 days.

For adipogenic differentiation, $1x10^3$ cells/cm^2 were cultured in a 6-well plate in adipogenic medium (GM, supplemented with 0.5 mM isobutyl-methylxanthine, 1 mM dexamethasone, 10 mM insulin, 200 mM indomethacin), with three weekly medium changes. Oil Red O staining (Thermo Fisher Scientific) was performed following manufacturer's instructions, as an indicator of intracellular lipid accumulation. Cells were washed with PBS and fixed in 10% formalin for 1 h. After washing with 60% isopropanol, cells were stained with Oil-Red O solution in 60% isopropanol for 5 min, rinsed with deionized water, and counterstained with hematoxylin for 1 min.

Osteogenic differentiation was induced by culturing $2.5x10^4$ cells/cm^2 in a 24-well plate in osteogenic medium [GM, supplemented with 50 mM ascorbic acid (Sigma-Aldrich) and 10 mM β-glycerophosphate (Sigma-Aldrich)], with three weekly medium changes. Cell differentiation was assessed by Alizarin Red staining, as an indicator of extracellular matrix calcification. For Alizarin Red staining, cultures were fixed in 70% ethanol, incubated with 2% Alizarin Red (Sigma-Aldrich) for 15 min, and rised with deionized water.

Chondrogenic differentiation was induced by culturing $2.5x10^4$ cells/cm^2 in a 24-well plate in chondrogenic medium [GM, supplemented with 1 mM dexamethasone, 100 mM pyruvate, 100 U/mL Insulin, 5 ug/mL Transferring, 50 mg/mL ascorbic acid (all chemicals from Sigma-Aldrich) and 10 ng/mL TGF-β1], with three weekly medium changes. To evaluate chondrogenic differentiation, plates were fixed in 4% paraformaldehyde and proteoglycan deposition was detected by 1% Alcian blue staining (Sigma-Aldrich).

For all three protocols, control experiments were performed in cells plated on the same conditions and maintained in GM for 21 days.

Imaging analysis

All images were capture dusing the Motic AE31 inverted phasemicroscope withan Moticam 2300 camera and the Motic Image plus 2.0 software.

RESULTS

Cell morphology in expansion phase

Right after seeding, cells in culture presented a heterogenic morphology, with some of them showing a fibroblast-like aspect, and others, oval shape (Fig. 2A). After the first passage, however, cells shape became predominantly fibroblastoid, presenting an elongated appearance (Fig. 2B). The fibroblast-like morphological aspect persisted until the conclusion of the experiments (Fig. 2C). Enrichment for DPSC was performed based on their adherent capacity to the plastic. DPSC colony organization was observed in all passages (Fig. 2A). These DPSC reached confluence of 80% in approximately three days. After the third passage, however, a longer time was need to DPSC reach confluence in culture.

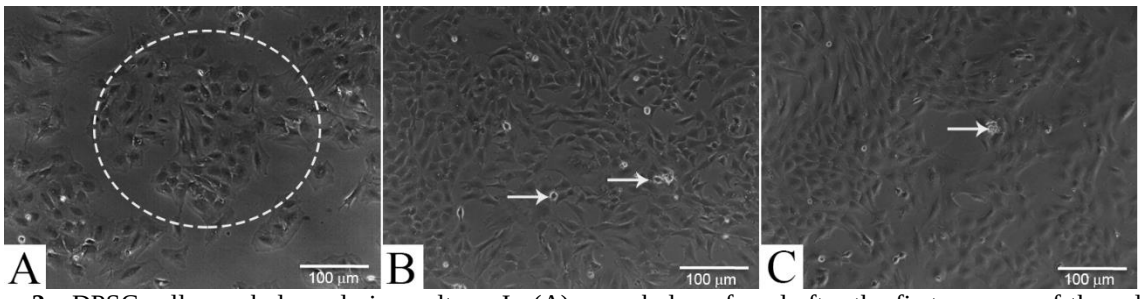

Figure 2 - DPSC cell morphology during culture. In (A), morphology found after the first passage of the cells. A heterogeneous cell shape was found, with cells presenting both fibroblastoid and round cell shapes. Cell clusters were also observed, suggesting the colony formingunit– dottedcircle. In (B), morphologic aspect observed at the second passage; and in (C) at the third passage. Cells were predominantly fibroblastoid, presenting an elongated appearance. Arrow indicates non-adherent round shape cells insuspension, discarded during medium replecement.

Phenotypic characterization DPSC

The immunophenotypic characterization revealed that the DPSC were positive for surface markers of nucleated cells such as HLA-ABC; and also for MS Cmarkers, such as CD54, CD90 e CD73. On the other hand, DPSC were negative for hematopoietic cell markers (CD11b/c, CD34 and CD45)(Fig. 3A-B). Additionally, RT-PCR results

revealed that the embryonic gene Nanog was expressed in DPSC during all three passages (Fig. 3).

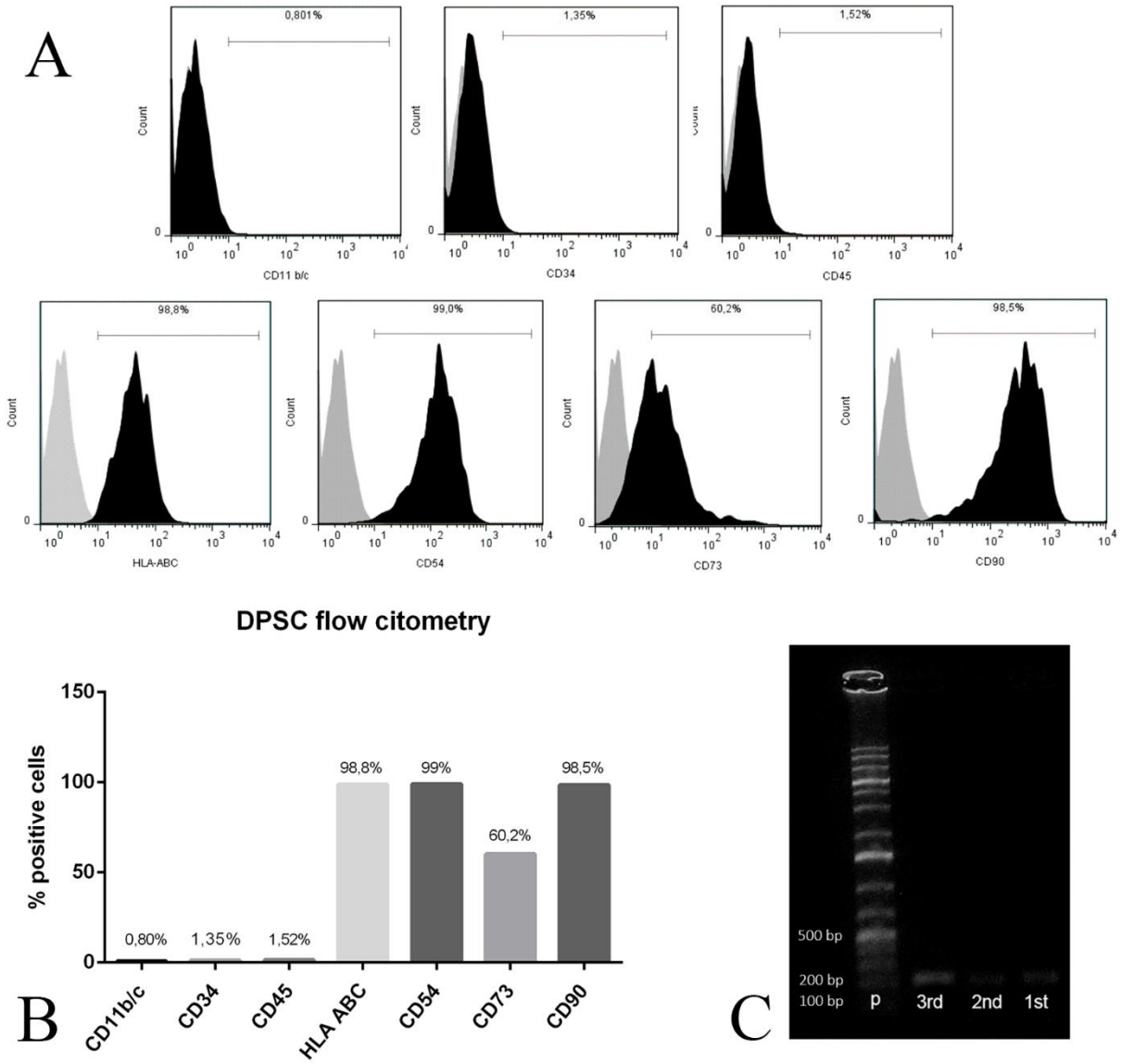

Figure 3.DPSC characterization. In (A) and (B),the percentage of DPSC staining indicated after cytometric flow for HLA-ABC, CD54, CD73, CD90, CD11b/c, CD34,and CD45markers.DPSC staining were positive for HLA-ABC, CD54, CD73 and CD90 markers; while negative for the CD11b/c, CD34 and CD45 markers. In (C), RT-PCR result indicating the expression of the embryonic gene Nanog during all passages of DPSC culture. P - 1kb plus molecular weightladder; 3rd – DPSC in the third passage; 2nd – in the second passage; and 1st – in the first passage.

Cell plasticity analysis

After three weeks of culture, the DPSC isolated using the proposed method from this work could successfully differentiate in all three cell fates: condogenic, osteogenic and adipogenic.

The DPSC osteogenic differentiation was confirmed by the alizarin red staining. The results revealed the deposition of mineralized nodules in the extracellular matrix of these cells, after 21 days of culture (Fig. 4A), which is characteristic ofosteblasts. DPSC cultivated in non-supplemented GM, were negative for this staining (Figure 4B).

After three weeks, DPSC cultivated under adipogenic condition could also be successfully converted in adipocytes (Fig. 4C). Differentiated cells were found as rounded shape, and presented a characteristic spread out pattern in the well (Fig.4C). Lipid droplets formed in the cytoplasm of differentiated adipocytes could be positively stained with oil red (Fig. 4C, arrows), confirming

the phenotype. No lipid droplets were found when DPSC were cultivated with GM (Fig. 4D).
Finally, DPSC cultured in the presence of condrogenic medium revealed proteoglycan deposition, confirmed by alcian blue staining (fig.4E and E'); while no positive staining was found in the negative control (Fig.4F).

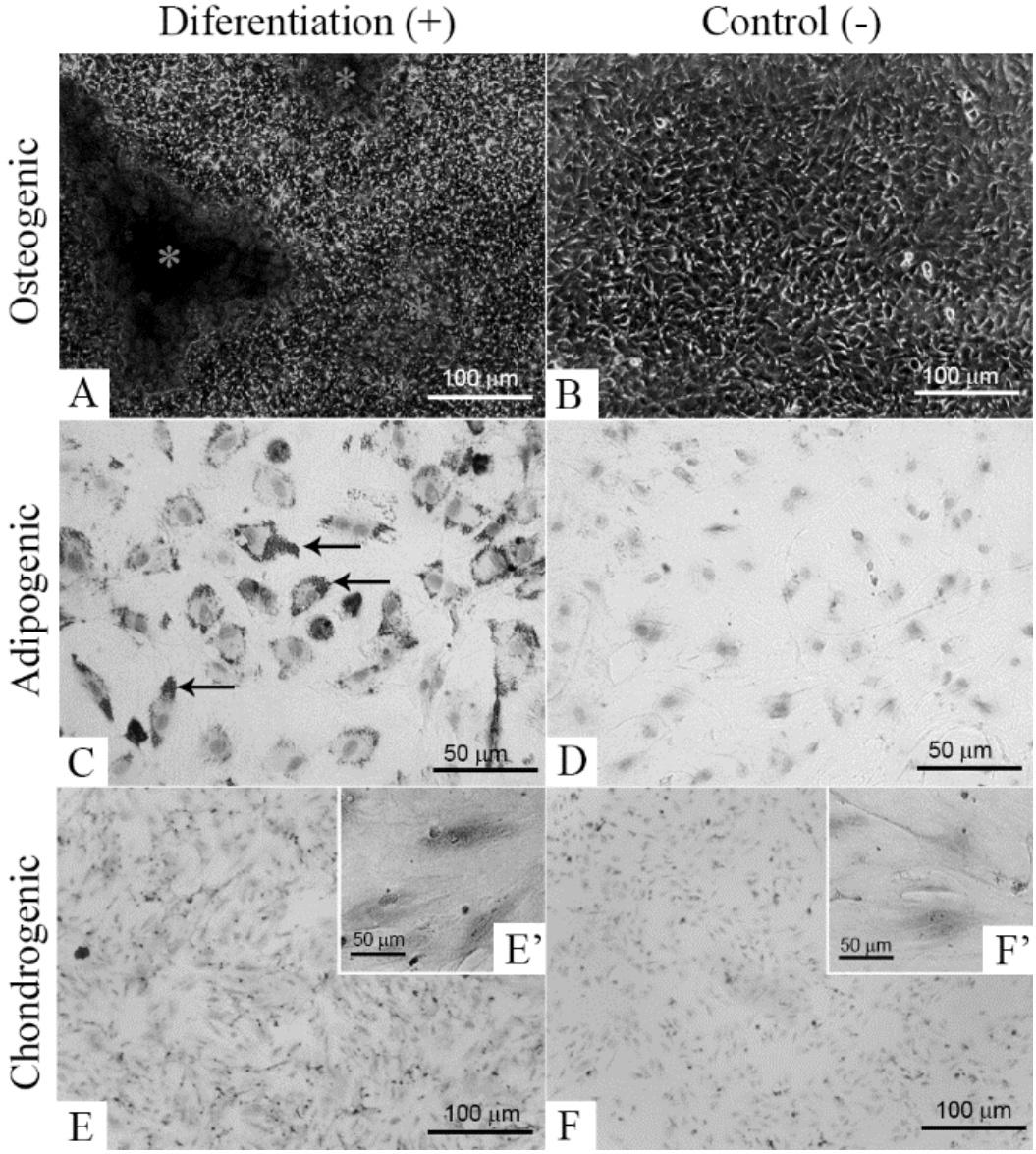

Figure 4. DPSC differentiation in osteogenic, adipogenic and chondrogenic cell fates. In (A), the presence of mineralized nodules stained with alizarin red (*) confirmed the DPSC differentiation in osteoblasts; and in (B), the negative control. In (C), lipid droplets stained with oil red (arrows) confirmed the positive adipogenic differentiation of the DPSC; and in (D), negative control. In (E), the positive staining of extracelular matrix protegly can by alcian blue, confirming the chondrogenic differentiation of the DPSC isolated in this work; in (E') zoom of (E); In (F), the negative control and in (F') zoom of (F).

DISCUSSION

The term "stem cell" can be applied to a diverse group of cells that share two properties: (1) a capacity for prolonged or unlimited self-renewal under controlled conditions; and (2) the potential to differentiate into a variety of specialized cell types (Dawson et al. 2014). The term "mesenchymal stem cell" was originally used for a hypothetical common progenitor, of a wide range of "mesenchymal" (non-hematopoietic, non-epithelial, and mesodermal) tissues (Caplan1991). The presence of MSC in a broad range of postnatal tissues is now widely accepted, supported by *in*

vitro assays and immunophenotyping (Dominici et al. 2006). MSC were originally named “bone marrow stem cells” by Friendenstein and co-workers (1966), grouping those stem cells that werenon-hematopoietic, adherent to polystyrene plates, and with a fibroblast-like morphology.

In this work, mesenchymal stem cells were isolated from dental pulps, using a particular method that allowed removing the pulp tissue directly from the apical foramen of the tooth.

Right after seeding, the DPSC isolated using this method were found to be adherent to the polystyrene plastic. However, adhesion properties are not sufficient to classify these purified cell population as MSC (Phinney et al. 1999). In fact, previous studies have been reported that fibroblasts, macrophages, and some hematopoietic cell lines can all adhere to the plastic, and also present MSC morphological characteristics after growth (Schrepfer et al. 2007; Bydloswki et al. 2009). Cell density can also be a factor that influences the capacity of MSC to adhere to the plastic: MSC plated in low-density conditions adhere faster to the plastic, compared to high-density conditions (Deans and Moseley 2000).

Additionally, DPSC from this work were not homogeneously found in the fibroblastoid-shape in the first passage. A homegeneous aspect of the DPSC, with the majority of the cells presenting a spindle/fibroblastoid morphology arranged in colony forming units, was observed in culture only after the third passage. Dental pulp connective tissue is a well known heterogenic cell population, what could be an explanation to our initial observations. This characteristic of finding fibroblast-like morphology of these cells only after a few passages was also reported in stem cells derived from bone marrow and deciduous teeth (Friendenstein et al. 1966; Gronthos et al. 2000; Gronthos et al. 2002; Jesus et al. 2011), suggesting this as a characteristic of MSC.

Another characteristic of the DPSC isolated in this work was that they initially reached confluence of 80% in approximately three days. After the third passage, however, a longer time was needed to reach confluence in culture. This observation suggested that successive passages could be inducing a decrease of the proliferation rate or even cell death in these cells. This characteristic was also described for MSC after several passages (Fehrer and Lepperdinger 2005).

Different methods of MSC isolation and expansion were reported thus far. The “Mesenchymal and Tissue Stem Cell Committee", of the International Society for Cellular Therapy, proposed the minimal criteria to define human MSC (Dominici et al. 2006). Following this criteria, MSC can be characterized by: (1) the capacity to adhere to the plastic; (2) the pattern of expression of surface markers known as “Cluster of Differentiation”, being positive for CD105 (Endoglin/SH2), CD73 (SH-3) and CD90 (Thy-1), and negative for CD45, CD34, CD14 or CD11b, CD79alpha or CD19 and HLA-DR surface molecules; and (3) the potential to *in vitro* differentiate in osteoblasts, adipocytes, and chondroblasts (Dominici et al. 2006; Kolf et al. 2007; Iacono et al. 2015). Although designed for human stem cells, the same designation has been referenced and used to characterize stem cells obtained from animal tissues.

The phenotypic characterization of the stem cells before the differentiation in specialized cell types can reveal the purity of the culture, as hematopoietic cells and fibroblasts could exhibit similar morphological characteristics *in vitro* (Bianco et al. 2001; Bobis et al. 2006; Ishii et al. 2005). Hematopoietic cells, for example, express the surface marker CD45 (Bobis et al. 2006; Pittenger et al. 1999), which can also be found in fibroblasts (Ishiiet al., 2005). The CD73 surface marker can either be expressed in fibroblasts or in MSC (Ishii et al., 2005). CD90 and CD54 were both found to be MSC exclusive (Covas et al. 2003).

In this study, CD90 was expressed in over 90% of the cells. This result strongly suggested that the cell culture was in fact a MSC culture. However, only 60.2% of the isolated cells were positive for CD37. According to Domicini and co-workers (2006) criteria, CD73 must be found in more than 90% of human MSC. Low expression of this particular surface marker was also observed in DPSC from humans third molar (Atari et al. 2012), suggesting that this could be a particular characteristic of DPSC. Curiously, the same authors have described a low expression of the CD90 marker in DPSC, what differs from our results. These discrepancies in surface marker expression patternmay be dueto:(1) the source of tooth/animal model; (2) the method of stem cell isolation;(3) the cell maintenance *in vitro;* and (4) most likely, the possibility of the presence of cell subpopulations, presenting phenotypic variations.

CD34 is a highly glycosylated integral surface protein. CD34 was found to be a regulator of the cytokine-dependent adhesion/differentiation and

proliferation characteristics of hematopoietic stem cells and endothelial progenitor cells (Gangenahalli et al. 2005). The DPSC from this work did not express this particular marker. The HLA-ABC antigens, also known as Major Histocompatibility Complex class I (MHC), are constitutively expressed in nucleated cells. The immunophenotypic characterization revealed that 98.8% of the DPSC isolated in this work were MHCI positive. Overall, the proposed method for stem cell isolation from dental pulp tissues resulted in a cell population expressing CD54, CD73, CD90, and MHCI markers; but not expressing the markers CD45, CD11 and CD34. Therefore, the immunophenotypic characterization of the DPSC cells isolated from rat incisors confirmed the purity of over 90% of the culture.

Following the multipotency criteria, DPSC were chemically induced to differentiate, after 21 days, into osteoblasts, chondroblasts and adipocytes. The differentiation of stem cells in osteoblasts may represent a breakthrough for tissue bioengineering. Studies have been demonstrating the DPSC potential to induce regeneration of critical-sized calvarial defects in mice (Moshaverinia et al. 2014; Menicanin et al. 2014); in canine and swine models, local implantation of DPSC, associated with β-TCP and HA/TCP scaffolds, successfully induced bone regeneration in critical-sized orofacial bone defects (Zheng et al. 2009; Kim et al. 2009).

CONCLUSION

Stem cells can be easily isolated from dental pulp using a simplified method, which give access to the pulp via the apical foramen. DPSC isolated using this method expressed MSC surface markers, but not the hematopoietic ones. DPSC multipotentiality profile was confirmed by the differentiation of these cells into osteoblasts, adipocytes and chondrocytes. According to the minimum criteria established by ISCT, DPSC isolated from rats incisors can be considered MSC. Given their plasticity, especially for osteogenic cell fate, this study opens perspectives for the evaluation of DPSC for bone tissue bioengineering and cellular therapy, aiming the replacement or regeneration of bone tissue.

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