Characterization of stem cells from the pulp of unerupted third molar tooth

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ABSTRACT

Context: Dental pulp stem cells (DPSCs) are the most diagnosed type of stem cells isolated from dental tissues. Previous studies demonstrate that tissues in earlier stages of development could be better stem cell resources for tissue engineering.

Aims: In this study, aiming at finding younger stem cell resources, we chose the pulp of human unerupted third molar teeth when the crown was completely formed and the roots had not begun their development, Nolla's 6th developmental stage (N6th).

Materials and Methods: Surgical removal of the third molar was performed by aseptic technique with minimal trauma. The tissues were digested enzymatically and the resulted single cells were cultured. Immunophenotypic characterization of the cells was done via immunocytochemistry, immunofluorescence, and flow cytometry assays. Adipogenic and osteogenic differentiation potential of these cells was examined and confirmed by histochemical staining and reverse transcription-polymerase chain reaction analysis.

Statistical Analysis Used: This study is descriptive.

Results: N6th -unerupted dental pulp cultured cells expressed DPSC markers: Vimentin, CD73, CD90, CD105, CD166, CD44, CD146, and STRO-1, but did not express hematopoietic cell markers: CD14, CD34, CD45, HLA-DR and were also negative for dentin sialoprotein negative showing an undifferentiated preodontogenic state. Adipocytes differentiated from N6th-DPSCs were positively stained with Oil-Red-O and expressed both early and late adipocyte specific genes. Formation of Alizarin-red positive condensed calcium-phosphate nodules accompanied by strong expression of two osteogenic mRNAs, exhibited osteogenic differentiation.

Conclusion: Based on the results of this study, we suggest that N6th-DMSCs are a viable choice for cryo-banking and future usage in regenerative therapies; however, more investigations are necessary before clinical application can commence.

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Adult stem cells (ASCs) with characteristics such as proliferation, differentiation and plasticity, and without ethical and legal concerns are promising resources for tissue bioengineering. To date, the most encouraging ASCs are "multipotent mesenchymal stromal cells (MSCs)," previously named as "mesenchymal stem cells."[1] These cells are multipotent cells with easy *in vitro* expansion and various differentiation potentials to the adipocyte, osteoblast,[2] chondrocytes, hepatocyte,[3] and neural cells.[4]

MSCs are mainly isolated from the bone marrow but can be obtained from different sources such as adipose tissue,[1] umbilical cord,[5] and dental pulp (dental pulp stem cells [DPSCs]).[6,7]

Dental tissues' mesenchyme is termed "ectomesenchyme" as it interacts earlier with the neural crest. Ectomesenchyme-derived dental stem cells do have characteristics akin to those from the bone marrow. Dental tissues are more specialized and do not undergo continuous remodeling compared with bony tissues, although DPSCs share many characteristics

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with BM-MSCs. Furthermore, dental-tissue-derived stem/progenitor cells are more committed to odontogenic rather than osteogenic development.[8] DPSCs are mostly obtained from human third molar teeth. These teeth, even before their eruption, are commonly extracted in dental clinics and extraordinarily their development initiates years after birth.[9] Previous studies demonstrate that tissues, which are younger and are in an earlier stage of development could be better stem cell resources for tissue engineering.[10,11]

In this study, aiming at finding younger stem cell resources for future regenerative purposes, we chose the pulp of human third molar tooth before eruption when the crown was completely formed and the roots had not begun their development, Nolla's 6th developmental stage (N6th). The tissue is a very rudimentary tissue with exceptional clinical availability. We have evaluated characteristics of N6th-dental pulp MSCs (N6th-DPSCs) after multipassaging and cryopreservation.

MATERIALS AND METHODS

Subjects and tissue sampling

The normal human unerupted third molar teeth, when the crown was completely formed and the roots had not begun their development, N6th third molar, were obtained from healthy young adults, who were referred to an oral and maxillofacial surgeon to remove their lower third molar teeth for orthodontic treatment [Figure 1].

After taking an informed written consent, surgical removal of the third molar was performed by aseptic technique with minimal trauma. The teeth were rinsed with sterile normal saline, and the pulp tissues were gently extracted from their apical portion, adjacent to flame. The tissues were transferred immediately to a cell culture laboratory in a caped sterile tube containing Roswell Park Memorial Institute (RPMI) 1640 media (Gibco/Invitrogen, Carlsbad, CA, USA).

Single cell preparation and culture

Tissues were minced (<1 mm^3) using a sterilized scalpel under an aseptic condition, and digested in the same volume solution of 3 mg/ml collagenase type I (Sigma, St. Louis, MO, USA) for 1 h at 37°C. To neutralize the collagenase action, an equal volume of standard growth medium was added to the mixture, and single cell suspension was obtained by passing the mixture through a 100-μm cell strainer (BD Falcon™, BD Biosciences, San Jose, CA, USA). It was then transferred to a 15 ml polypropylene tube (Greiner, Frickenhausen, Germany) and centrifuged for 10 min at 375 *g*.

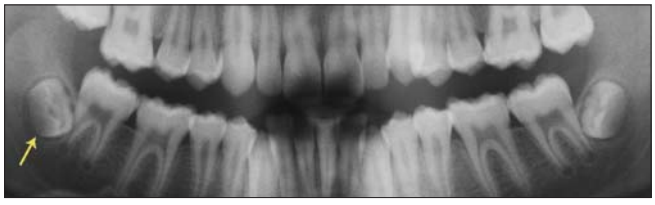

Figure 1: Patient radiograph: Lower right third molar at Nolla's 6th developmental stage (arrow)

After supernatant decanting, the pelleted cells were resuspended in standard growth medium: Dulbecco's Modified Eagle Medium (DMEM) low glucose (1 × liquid, Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco/Invitrogen), 1% L-glutamine (Invitrogen), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). The homogenized single cell suspension was seeded into a T25 cm^2 tissue culture-treated flask (Nunc, Roskilde, Denmark) and cultured in 6 ml standard growth medium at 37°C in 5% CO_2 in a humidified incubator. Non-adherent cells were removed by changing the medium after 24 h. Plastic adherent cells were continuously propagated until reaching confluence and being prepared for the first passage.

Cryopreservation of cells

After each harvesting, an aliquot of final homogenized cell suspension was centrifuged for 8 min at 1000 *g*, and the pelleted cells were resuspended in 1 ml freezing medium: 10% Dimethyl sulfoxide (DMSO) (Sigma), 30% FBS, 1% penicillin-streptomycin, in DMEM. The suspension was then transferred into cryotube (Nunc) and directly stored in –70°C. After 24 h, the cryotubes were transferred to liquid nitrogen for long term storage.

To prepare the cryopreserved cells for culture, after thawing at room temperature, they were transferred to a centrifuge tube and an equal volume of appropriate medium (e.g. standard growth medium) was added drop by drop to remove DMSO from the cells slowly. Then, the homogenized suspension was centrifuged at 1200 *g* for 5 min. After supernatant decanting, the cells were resuspended in the same medium.

A number of harvested cells from the fifth and seventh passages which had been cryopreserved for 6 months were defrosted and let to proliferate. After one more passage (for flow cytometric assay) producing enough cells, their immune phenotype and differentiation potential was studied.

Immunocytochemistry

After the third and sixth passages, the slides were prepared by two methods: (A) Cells were sub-cultured into 60 cm^2 petri dish, with sterile glass slides located inside (to allow the adherent cells to grow on them), in standard growth medium, and incubated at 37°C in 5% CO_2 in a humidified atmosphere. After 2 weeks, the slides were brought out and fixed for 10 min in cold acetone. (B) An aliquot of harvested cells was washed and resuspended in Phosphate Buffered Saline (PBS). Then, a few suspended cells were concentrated on each of the pretreated slides

by cytocentrifuging for 6 min at 550 *g*. The slides were air dried and fixed for 10 min in cold acetone. They were then washed in PBS for 10 min (this was repeated after each step till mounting), and immersed in 1% H_2O_2/PBS for 30 min to quench the endogenous peroxidase activity. Non-specific back-ground was blocked in 0.1% normal goat serum in PBS. Without washing, the specimens were incubated for 75 min at room temperature with primary antibodies: Mouse monoclonal (IgG) anti-human vimentin, anti-HLA-DR (Dako, Glostrup, Denmark) and anti-human CD146 (Abcam, San Francisco, CA, USA).

For immunoperoxidase staining, universal LSAB™ two kits (Dako) was used. In brief, the slides were incubated with secondary antibody: Biotin-conjugated goat (IgG) against mouse immunoglobulin, followed by 30-min incubation with HRP-strepavidin complex and visualized by adding one drop 3,3'-diaminobenzidine (chromogen) and 1 cc buffer with H_2O_2 (katalisor) (5-10 min). Subsequently, the specimens were counterstained with hematoxylin and mounted in 100% ethanol.

Immunofluorescent labeling

The slides containing cells at sixth passage (as described in the previous part, A and B) were washed 3 times with PBS, then blocked in 10% normal (goat or mouse) sera and again washed. They were incubated for 60 min with primary antibodies: Mouse monoclonal (IgM) anti-human STRO-1, goat polyclonal (IgG) anti-human DSP (dentin sialo protein) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) which was diluted (1:40, Optimal concentration was determined by titration.) in PBS with 1.5% normal blocking serum and then washed 3 times with PBS for 5 min each. Treating with diluted (1:40) secondary antibodies: Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM, tetra methyl rhodamine isothiocyanate -conjugated mouse anti goat IgG (Santa Cruz Biotechnology Inc.) was performed in a dark chamber for 45 min and terminated by washing 3 times with PBS. In addition, counterstaining with Evance-blue was done. Then, all the specimens were mounted with 90% glycerol in PBS and observed under a florescence microscope with appropriate filters: 495 and 550.

Flow cytometric analysis

Subcultures of passage 5 were used for flow cytometric analysis. The cells were trypsinized, washed and resuspended PBS. Then, the cells were incubated 30 min in a dark environment with following anti-human antibodies: CD45-FITC, CD34-FITC, CD14-FITC, CD166-phycoerythrin (PE), CD44-PE, CD73-PE (BD Pharmingen, BD Biosciences, San Jose, CA, USA), CD90-FITC and CD105-PE (AbD Serotec, Kidlington, Oxford, UK). Mouse IgG1-PE and IgG2a-FITC (AbD Serotec, Kidlington, Oxford, UK) were used as the isotype controls. Cells were analyzed by BD FACS-Calibur instrument. Data was analyzed using WinMDI software (BD Biosciences).

In vitro differentiation assay

Adipogenic differentiation

After four passages, the cells were seeded in 35 cm^2 petri dish with a sterile slide located inside, and cultured in 7 ml Complete Mesencult® Adipogenic Medium: 10% Mesencult® Adipogenic Stimulatory Supplements in Mesencult® MSC Basal Medium (human) (Stem Cell Technologies Inc., Vancouver, BC, Canada) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen), at 37°C in 5% CO_2; and medium change was performed regarding the manufacturer's guidelines. After the formation of cytoplasmic vacuoles, the cells were harvested and used to prepare the slides by cytocentrifuging. Then the specimens were stained with a lipophilic red dye, Oil-Red-O: The cells were fixed in calcium-formal (4% formalin, 1% calcium) (Merck, Darmstadt, Germany), rinsed in 70% ethanol for a second, and stained with sufficient filtered Oil-Red-O staining solution (0.5 g powder (Sigma)/60% isopropanol) to cover the specimens for 10 min. After rinsing in 70% ethanol and distilled water, hematoxylin counterstaining was performed.

The same adipogenic induction was performed on the cells after eight passages, but they were cultured in 6-well plate (Nunc) (5×10^4 cells/ml, 3 ml/well) and kept for 3 weeks to have further adipogenic development. Also, RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) was performed for detection of peroxisome proliferator-activated receptor gamma, transcript variant 2, (*PPAR-γ2*, mRNA), which encodes PPAR-γ protein, a regulator of adipocyte differentiation; and adipocyte protein 2 (*ap2*, mRNA) or FABP4 that encodes fatty acid binding protein found in the adipocytes.[12]

Osteogenic differentiation

After the fourth passage, the cells were cultured in NH-osteoDiff medium (MACS® NH Stem Cell Media, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) with 1% penicillin/streptomycin, (1 ml/well) (Nunc) at 37°C in 5% CO_2 for 14 days, and every 3rd day half of the medium was exchanged.

Osteogenic differentiation was assessed by two tests: (1) RT-PCR was done for detection of *osteopontin* and *col1α1* mRNA. Osteopontin (expressed in bone, role as linking protein), also known as secreted phosphoprotein 1 and bone sialoprotein I, is an extracellular structural protein of the bone. Col1α1 encodes pro-alpha1 chains of type I collagen that is a fibril-forming collagen found in most connective tissues and is abundant in the bone.[13]

Alizarin red staining was performed in order to detect the *in vitro* formed mineralized matrix. Cells in the wells were rinsed with PBS and fixed in methanol for 10 min. Subsequently, they were stained for 10 min at room temperature with Alizarin red, which was prepared by

dissolving 1 g powder in 100 ml 25% ammonia water. Finally, they were washed extensively with deionized water and air dried.

RT-PCR

RT-PCR was performed to determine the expression of adipocyte-specific genes and osteoblast marker genes in the induced cells. Total RNA was extracted from the cultured cells utilizing TriPure Isolation Reagent (Roche Applied Science, Mannheim, Germany) in three steps and according to the manufacturer's instruction. Then, a spectrophotometer (Bio-Rad, Hercules, CA, USA) was used to quantify the total RNA yield. In each sample extracted RNA was reverse-transcribed into first strand complementary DNA (cDNA) by using the Sensiscript® Reverse Transcription kit (Qiagen, Hilden, Germany), according to the manual's instruction.

Each polymerase chain reaction (PCR) was performed using cDNA on Mastercycler® gradient PCR Thermal Cycler (Eppendorf AG, Hamburg, Germany) in a reaction mixture which contained PCR master mix consisting of forward primer, reverse primer (Metabion international AG, Martinsried, Germany) [Table 1], dNTP mixture, ×10 PCR buffer, $MgCl_2$, Taq DNA polymerase (Fermentas, Life Science, EU), and distilled water. For PPAR-γ2 and ap2, after initial denaturation at 95°C for 15 min, 35 cycles of denaturation for 30 s at 95°C, annealing for 30 s at 60°C, and extension for 1 min at 72°C were carried out and the PCR was terminated by a final extension at 72°C for 7 min. Amplification of Col1αI and osteopontin messages was performed for 40 cycles at 95°C for 30s, 57°C for 20s and 72°C for 30s following initial denaturation at 95°C for 10 min and PCR was terminated by a final extension at 72°C for 10 min. PCR products were analyzed by 2% agarose gel electrophoresis, visualized by ethidium bromide staining and photographed.

RESULTS

Culture characteristics of N6th-DPSCs

The isolated cells showed proper ability to adhere to plastic substratum, formed single cell derived colonies, and had typical fusiform fibroblast-like morphology [Figure 2]. N6th-dental derived cells showed proper proliferative capacity even in the 20th passage [Figure 2a] and without any sign of spontaneous differentiation or morphological evidence of cellular senescence.

Immunophenotypic characterization of N6th-DMSCs *in vitro*

Immunocytochemistry study on the N6th-dental derived culture expanded cells revealed the expression of vimentin, an intermediate filament protein typically associated with mesenchymal cells [Figure 3a], and also CD146 [Figure 3b]. Besides, this study demonstrated absence of HLA-DR [Figure 3c].

Immunofluorescent studies depicted expression of STRO-1 [Figure 3d]. These cells were also found to be negative for DSP, which is suggestive of an undifferentiated phenotype [Figure 3e]. Thereafter, immunophenotyping was continued (after six or more passages and 6th month cryopreservation) using a large panel of antibodies known to be associated with human MSCs. The cells strongly expressed CD73, CD90, CD105, CD166, and CD44. They were negative for the hematopoietic lineage markers CD14, CD34, and CD 45 [Figure 4].

Multipotent differentiation of the cells *in vitro*

To evaluate the odonto/osteogenic and adipogenic differentiation ability of the cells before and after cryopreservation, the cells from the fourth passages before and after 6 months of cryopreservation were used. After 14 days of induction, the cells cultured in adipogenic medium exhibited an enlarged cell body containing abundant small uniform cytoplasmic vacuoles, positively stained with Oil-Red-O [Figure 5a and b]. However, typical lipid clusters formed when the cells were maintained under the adipogenic condition for 21 days [Figure 5c]. Moreover, the cells were able to upregulate both early (PPAR-γ2) and late (ap2) adipocyte specific genes as demonstrated by RT-PCR [Figure 5d].

The potential of the cells to differentiate into osteoblasts was revealed after 14 days of culture in osteogenic medium. This was evidenced by the formation of Alizarin red positive condensed calcium-phosphate nodules, which were scattered over the adherent layer [Figure 5e], and also by generous expression of col1α1 and osteopontin mRNA detected through RT-PCR [Figure 5f].

Table 1: PCR primer sequences and their amplified product size

Gene	Primer sequence		Product length (bp)
β-Actin[a]	5´-ATCATGTTTGAGACCTTCAA-3´	Forward	317
	5´-CATCTCTTGCTCGAAGTCCA-3´	Reverse	
PPAR-γ2	5´-TTCTCCTAT TGACCCAGAAAGC-3´	Forward	307
	5´-CTCCACTTTGATTGCACTTTGG-3´	Reverse	
ap2	5´-GCCAGGAATTTGACGAAGTC-3´	Forward	107
	5´-TGGTTGATTTTCCATCCCAT-3´	Reverse	
Osteopontin	5´-TTCCAAGTAAGTCCAACGAAAG-3´	Forward	181
	5´-GTGACCAGTTCATCAGATTCAT-3´	Reverse	
Col1αI	5´-AAGCCGAATTCCTGGTCT-3´	Forward	195
	5´-TCCAACGAGATCGAGATCC-3´	Reverse	

[a]β-Actin is a relatively stable cytoskeletal protein that is ubiquitously expressed and serves as a positive control, PCR=Polymerase chain reaction, bp=Base pair

DISCUSSION

In the present study, we performed a descriptive study to evaluate the presence of stem cells in the N6th-Nolla wisdom tooth which is a very young adult tissue resource and hopefully can serve for future regenerative purposes.

The cells derived from N6th-Nolla third molar tooth were totally vimentin positive, revealing their mesenchymal identity. The uniform expression of CD73, CD90, CD105, CD166, and CD44 accompanied by the absence of CD14, CD34, CD45, and HLA-DR after multiple passaging and cryopreservation, confirmed the "stability" of these standard positive and negative MSC markers.[14,15] The expression of

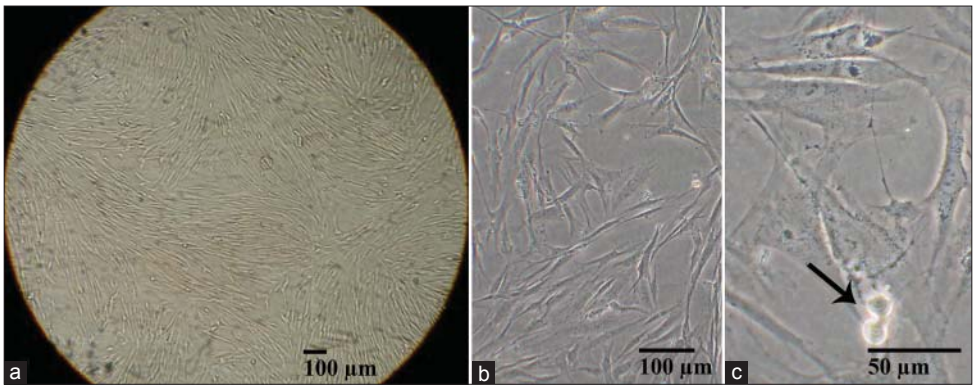

Figure 2: Proliferative N6th-DPSCs in standard growth medium. Confluent cell culture before passaging (a); typical morphology of adherent fibroblast-like cells (b); small, round and refringent cells during division (arrow) (c)

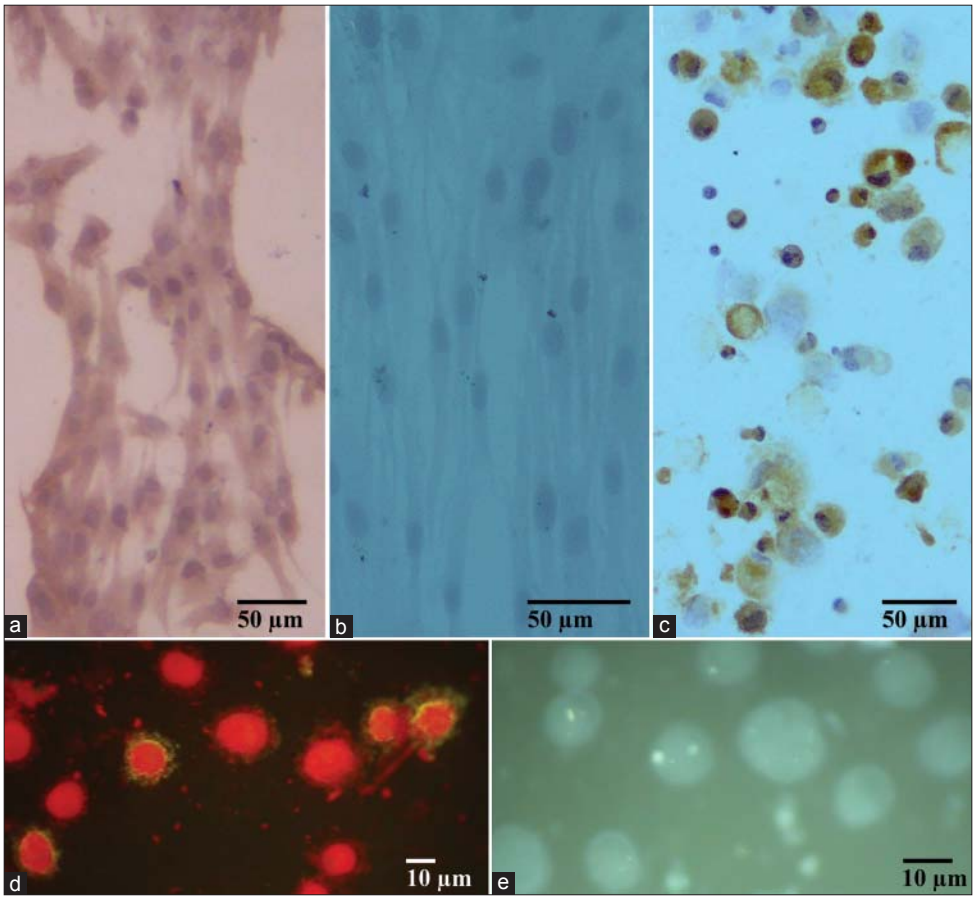

Figure 3: Immunophenotypic characterization of N6th-DPSCs *in vitro*: Immunocytochemistry based on immunoperoxidase reactivity (a-c), immunoflourescent labeling (d, e). Expression of vimentin (cytoplasms are brown) (a), and absence of HLA-DR (b) was shown for all cells, adhering on slides during their culture in standard growth medium. Other slides were prepared with harvested cells. Most of N6th-DPSCs expressed CD146: Cells with brown membrane (c). Some of them were STRO-1 positive (green fluorescence) (d), and none of them expressed DSP (no red fluorescence) (e). Evence-Blue counterstaining visualized cell nuclei (red in d, blue in e)

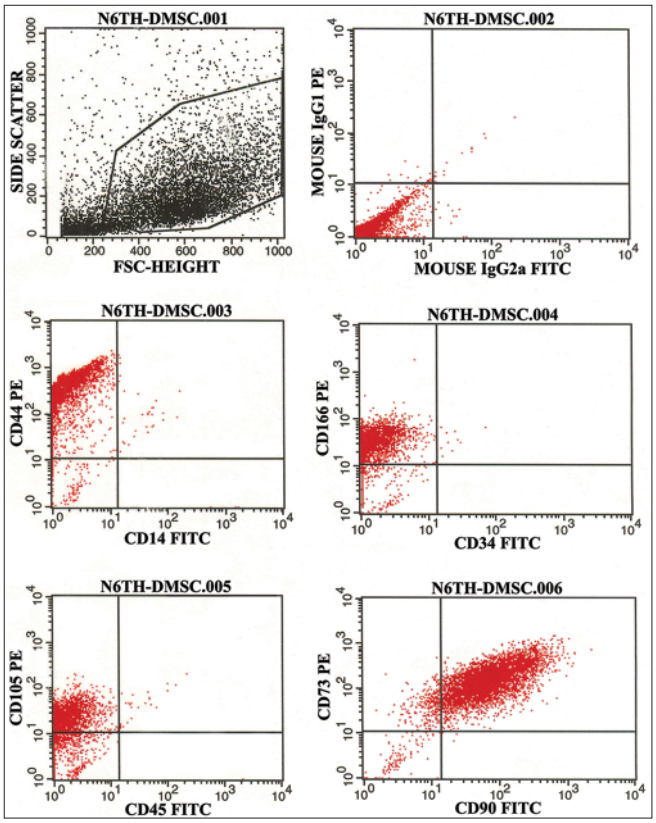

Figure 4: Immunophenotypic characterization of N6th-DPSCs *in vitro*: Flow cytometry. Flow analysis of these dental MSCs revealed expression of CD44 (96.76%), CD166 (89.36%), CD105 (77.03%), CD90 (96.43%), and CD73 (96.43%), but was negative for surface molecules CD14, CD34, and CD45

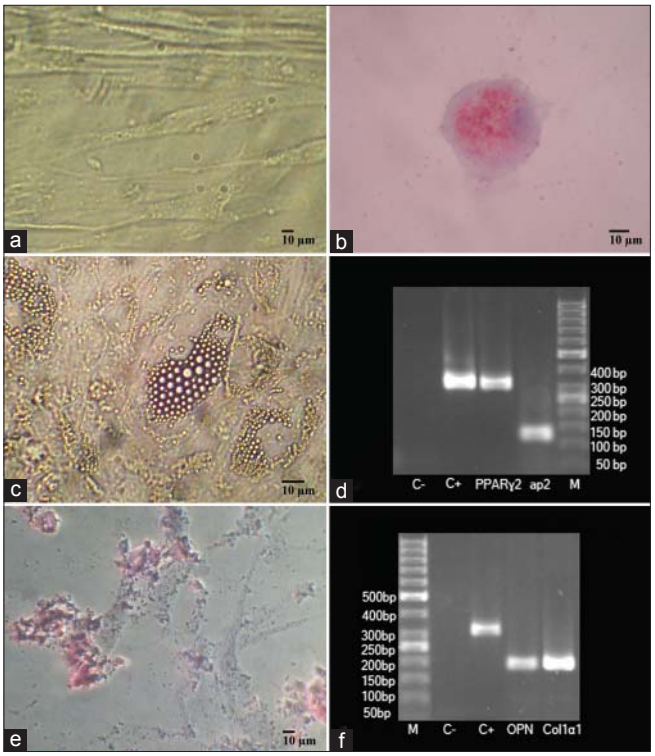

Figure 5: Adipogenic (a-d) and osteogenic (e, f) differentiation potential of N6th-DPSCs *in vitro*. Accumulation of lipid vacuoles in treated cells after 2 weeks induction (a) was confirmed by staining with Oil-Red-O (b). After 21 day culture in adipogenic medium typical lipid clusters were formed (c). Expression of PPAR-γ2 and ap2 (indicative of adipogenic activity) was revealed by RT-PCR (d). Adherent layer of cultured N6th-DPSCs is shown with Alizarin red staining as a measure of calcium accumulation after 2 weeks of induction (e). RT-PCR showed a significant expression of col1γ1 and osteopontin (indicative of osteogenic activity) (f). bp: Base pair, M: Markers, C–: No RNA, C+: γ-Actin, OPN: Osteopontin

STRO-1 and CD146 on these cells is in accordance with previous findings indicating that these two are identified as early MSC markers and both are intrinsically present on perivascular cells (e.g., pericytes) *in situ*. Thus, they infer a possible perivascular niche for this stem cell population.[16] Further, the absence of DSP on these cells is an indicative of undifferentiated pre-odontogenic cells because this marker is expressed by odontoblasts, pre-ameloblasts and other differentiated cells (including osteocytes, osteoblasts, cementocytes, cementoblasts and fibroblasts) of developing dental, periodontal and alveolar bone tissues.[17]

Thus, as perceived from our results, such antigen expression can be indicative of dental MSCs. All of these indicate a common feature displayed by various mesenchymal stem cell populations *in vitro*, being colony forming units-fibroblastic. Considering all these, we can claim that the cells we isolated are stem cells from mesenchymal origin and from now we mention them with N6th-DPSCs.

Practically useful stem cells must be able to maintain proliferative and differentiation characteristics after cryopreservation. Only in this condition, they can be stored for future therapies designed for tissue repair upon patient's needs. It is noticeable that cryopreservation of cells and tissues has recently been significantly improved.[18] In one study on cryopreserved tissue, the samples of minced periodontal ligament[10] and another on 1-month cryopreserved DPSCs followed by prolonged culturing[19] revealed that dental stem cells can maintain their potential after such procedures. Recently, it has been reported that DPSCs and their osteoblast differentiated cells after long-term (2 years) cryopreservation are still capable of quickly restarting proliferation and the production of woven bone (*in vitro*) and remodeling into adult lamellar bone (*in vivo*) in a manner similar to that of fresh cells.[18] We determined the differentiation potential of N6th-Nolla wisdom tooth cells when they had passed 6 months of cryo-storage and a relatively long time proliferation. The interesting point was that these cells after cryopreservation were not only able to demonstrate their differentiation potential, but also there was no significant alteration in their proliferation ability after 20 passages as it took approximately the same time for them to become confluent and ready for passaging in the 4th and 20th passages.

DPSCs, isolated from the pulp tissue of wisdom teeth exhibit a higher proliferation rate compared with BM-MSCs (bone

marrow mesenchymal stromal cells) *in vitro*. It is presumed that this can be attributed to the developmental state of the respective tissues because the third molars are the last permanent teeth that fully develop and erupt and therefore, they are at an earlier stage of development compared to the adult bone marrow.[6] More recently, the novel populations of MSCs have been obtained from the developing root of the impacted third molar. These cells not only proliferate significantly more than those in the mature pulp, but also have a higher differentiation capability both *in vitro* and *in vivo*.[20-22] These confirm that tissues at more primitive stages of development could be a better stem cell resource for tissue engineering.

Therefore, we used the ectomesenchymal soft tissue of the third molar tooth at N6th because it is not only usually the most primitive tooth excised in routine clinical (oral and maxillofacial) operation, but it can also be obtained easily and safely, and provide plenty of high potent stem cells. Nowadays, in comprehensive orthodontic treatment performed increasingly and mostly in adolescence, last molar tooth removal (before or during treatment) is commonly ordered.[23,24] So early developing tissues are more accessible in daily dental clinical practice now. Since most human tissues at the developing stage are not clinically available for stem cell isolation, this is an exceptional opportunity afforded by the naturally delayed formation of third molar teeth. The surgical removal of the third molar tooth in this stage is relatively easy and less traumatic because the crown is formed and roots which can complicate the removal process do not exist. Moreover, extraction of the desired soft tissues from their surrounding hard tissues (crown and socket) can be done quite simply by an explorer at most, whereas just obtaining mature pulp from the pulp chamber of the tooth (after root formation) at least necessitates cutting the crown with dental burs, which may itself be harmful for the cells.[21]

In view of this study, we have concluded that surpassing clinical availability of developing - N6th-third molars and the considerable ability of N6th-DPSCs to provide a large number of stem cells, along with expressing correct surface antigens and great differentiation potential after fairly long-term proliferation and cryopreservation render this ancestral population of DPSCs a viable choice for cryo-banking and future usage in regenerative therapies.

Nevertheless, more comparative and wider investigations for further identification of N6th-DPSCs and *in vivo* studies to assay their real regenerative potential in physiologic condition are necessary before clinical application can commence.

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