Original Article

Isolation and Evaluation of Dental Pulp Stem Cells from Teeth with Advanced Periodontal Disease

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Abstract

Introduction: Successful isolation of mesenchymal stem cells from waste tissues might be extremely promising for developing stem cell-based therapies. This study aimed to explore whether cells retrieved from teeth extracted due to advanced periodontal disease present mesenchymal stem cell-like properties.

Methods: Pulp cells were isolated from 15 intact molars and 15 teeth with advanced periodontal disease. Cell proliferation and markers of mesenchymal stem cells were evaluated.

Results: Based on the RT-PCR and agarose gel electrophoresis, nucleostemin, Oct-4 and jmj2c, but not Nanog, were expressed in undifferentiated mesenchymal stem cells of both groups. Interestingly, diseased pulp exhibited higher gene expressions although it was not statistically significant. The average percentage of BrdU positive cells in the diseased group (84.4%, n = 5) was significantly higher than that of the control group (65.4%, *n* = 5) (*t*-test, *P* = 0.001).

Conclusion: Our results indicate the successful isolation of mesenchymal stem cells from the pulp tissue of hopeless periodontally involved teeth.

Keywords: Dental pulp, mesenchymal stem cell, isolation, periodontitis

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Introduction

Stem cell biology has become a notable field for better understanding the process of tissue regeneration and provides important implications in various pathologic conditions including medical and dental care.[1] Mesenchymal stem cells isolated from bone marrow are the most intensively studied adult stem cells. Since the procedure for harvesting bone marrow stem cells is painful, invasive and causes complications in up to 30% of patients, the search for alternative stem cell sources has been started.[2]

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Postnatal stem cells with the property of self-renewal, proliferation into more specialized cells and differentiation into multiple cell lineages have been isolated from the dental pulp.[3] Dental pulp stem cells (DPSCs) are potentially accessible and easy for identification and isolation. They are able to differentiate into a variety of cell types, including odontoblasts, *in vitro*[4,5] and form dentin/ pulp-like complexes *in vitro*.[6,7] Moreover, DPSCs are going to be used in cellular therapy for some neurologic diseases through the secretion of neuroprotective factors such as vascular endothelial growth factor and fibroblast growth factor.[8,9] The mesenchymal stem cells of dental pulp origin may be useful in treating severe ischemic conditions of the heart, brain, or the limbs.[10] So, they may provide a good prospective in future regenerative dental and medical treatment.

DPSCs have been chiefly isolated from third molars.[11] However, there are some limitations in this regard. Third molars can be very difficult to clean, and therefore often get decayed. Moreover, some of them need surgical procedure and sectioning to be removed so the tooth does not remain intact for stem cell isolation.[12] On the other hand, some people miss the third molars. In a study by Liu *et al*. the percentage of males who missed one or more third molar germs was 37%, while the figure was 24.6% for females.[13]

Mild periodontal diseases affect an estimated 75% of adults in the United States. However, 20% to 30% of adults suffer from more severe forms of the disease. Extraction of teeth with severe attachment loss is common practice. Due to notable bone loss, this kind of teeth can be extracted easily and with a minimum degree of surgical trauma. Moreover, elderly people who

are at risk of different diseases who may need stem cell based therapies are more likely to suffer from periodontal problems.[15] So, these hopeless teeth may be a good source for mesenchymal stem cell isolation.

In the present study, we hypothesized that the pulp cells residing in pulp clinically diagnosed with severe periodontitis may still have mesenchymal stem cell potential similar to healthy pulp cells.

Materials and Methods

Isolation and culture of human dental pulp stem cells

Pulp tissues were obtained from freshly extracted teeth of 30 patients (17–55 years of age) recruited from Kerman Dental School. In this experimental study, informed consent was obtained from the patients after receiving approval by the Institutional Ethics Committee of Kerman University of Medical Sciences (Code: K/88/220). The characteristics of each sample are shown in Table 1.

The teeth were immersed in PBS solution and transported to the cell culture laboratory on ice. Pulp tissue was extracted and digested in a solution of 1 to 3 mg/mL collagenase/dispase for 20 minutes at 37°C and then centrifuged at 1200 rpm for 5 minutes. Cell suspensions were seeded in 60 mm culture dishes containing α-MEM; with 20% fetal bovine serum (FBS), 100 U/mL penicillin-G, 100 µg/mL streptomycin and 1 µg/mL amphotrypsin B and incubated at 37°C in 5% CO_2. The medium was replaced every three days. The cells were passaged 1:5 with 0.25% trypsin/1 mM EDTA every five days.

Differentiation of hDPSCs

Osteogenic differentiation

The hDPSCs were ready, with the exception that 1 nmol/L dexamethasone (Sigma), 20 mmol/L b-glycerolphosphate (Sigma), and 50 ng/mL L-thyroxine sodium pentahydrate (Sigma) were supplemented in the DPSC medium. The culture was incubated at 37°C and 5% CO_2 for another 22 days, with medium changes three times per week. As control, DPSC medium alone was used. At the end of the period, the DPSCs were fixed in 10% buffered formalin for 10 minutes and stained with alizarin red S (Wako, Osaka, Japan) for 15 minutes at room temperature so that the mineral matrix of the bone could be observed.

Chondrogenic differentiation

DPSCs were centrifuged in a 15-mL conical tube at 1,000 rpm for 5 minutes. The pellet was maintained in DPSC medium supplemented with 10 mg/mL ITS-X (Invitrogen), 5.35 mg/mL linoleic acid, 1.25 mg/mL bovine serum albumin (BSA), 1.0 mg/mL dexamethasone, and 10 ng/mL TGF-b3 (Chemicon, Billerica, MA). The medium was replaced every two to three days for four weeks. As control, DPSC medium alone was used. The pellets were then fixed in 4% paraformaldehyde (PFA) PFA overnight and paraffin-embedded sections (4 – 5 mm) were stained with Alcian blue.

Flow Cytometry

DPSCs were detached by 0.5 mL of % 0,25 trypsin and washed with PBS followed by incubation with 1 µg of conjugated CD34/PE(555822), CD73/FITC(561254), CD90FITC(555595), and CD105/PE(560839) primary antibodies or isotype-matched control immunoglobulins (all from BD PharMingen, USA) at 4°C for 30 minutes. Fluorescence intensity was measured on a FACSCalibur flow cytometer (BD, USA), and data were analyzed using Cellquest software (BD, USA).

RNA extraction and construction of complementary DNA (cDNA)

Using RNX-plusTM kit (CinnaGen, Iran), according to the manufacturer's protocol,total RNA was extracted from DPSCs. 0.5 µg of the RNA was treated with RNase-free DNase I (Fermentas, Litany) to remove residual contamination with genomic DNA. Total DNA was mixed with 0.2 µg of random hexamer and heated at 70°C for 5 min. The mixture was immediately chilled on ice for 5 min followed by the addition of 6.5 µL of a reverse transcription mixture prepared in a total volume of 19 µL containing 4 µL of 5 × reaction buffer, 2 µL of 10mM dNTP, 0.5 µL RNase inhibitor. The mixture was incubated at 25°C for 5 min and then 1 µL M-ML V RT was added. The cDNA-synthesis reaction was performed at 42°C for 60 min.

Polymerase chain reaction and agarose gel electrophoresis

Subsequent PCR was performed as follows: 1 µL CDNA, 1.5 mM MgCl2, 200 µM dNTP, 0.4 µm of each specific primers and 1.25 unit/25 µL reaction Taq DNA polymerase (Cinnagen, Iran). Amplification was undertaken by initial at 94°C for 5 min, denaturation at 94°C for 30 seconds, Annealing at 55°C for 30 seconds, extension at 72°C seconds for 30 seconds and final extension at 72°C for 5 min. Each experiment was repeated

Table 1. The sequences of primers designed and used for regular RT-PCR experiments.

Name Ref Seq.	Primer sequence	PCR product size
ACTB NM_001101	F: GGACTTCGAGCAAGAGATGG R: GACAGGACTGTGTTGGCGTA	237bps
NONOG NM_024865	F: TGATTTGTGGGCCTGAAGAA R: AGTGGGTTGTTTGCCTTTGG	155bps
NUCLEOSTEMIN NM_014366	F: GTGATTGAAGCCTCCGATGT R: AGCCAGCTCTCCAAAGGCTC	167bps
OCT4 NM_002701	F: AGTGAGAGGCAACCTGGAGA R: TTACAGAACCACACTCGGACC	120bps
JMJD2C NM_015061	F: GGTCCCAGAAGTTCGATT R: ATCCAGTGTTCCTACGGT	148bps

three times.

Specific primer pairs (Table 2) were designed using Primer3 software written by Whitehead Institute and oligonucleotides were synthesized by Isogen (Netherland). β-actin gene expression was used as an internal control (Table 1). PCR products were run on a 1.5% agarose gels using electrophoresis. In order to visualize the PCR products, gels were stained using ethidium bromide, documented using G BOX HR (Renishaw Plc, UK), and scored by Gene Tools software (Syngene, UK).

Cell proliferation investigation by BrdU test

Each of the considered cells for the experiment was grown on the three plates with six boxes in the free serum cell culture. After 24 hours, normal cell culture (BrdU roche) containing Bromodeoxyuridine was replaced. After 24 hours, investigation was done according to the Kit brochure (Invitrogen, Ca). The cooperation of BrdU in the proliferation was determined by monoclonal antibody, and then by addition of the streptavidin peroxidase and diaminobenzidine (DAB) to the cell culture the positive BrdU cells became visible. At the end, five random areas were chosen and the percentage of BrdU positive cells were determined by light microscopy (Nikon).

Statistical Analysis

Data are presented as means ± standard deviation. The differences in mean values of doubling times were evaluated by the t test after evaluation of variances (Microsoft Excel).

Results

Fifteen teeth were recruited in each of the control and diseased groups. There was no significant difference in the average age between the two groups.

In order to isolate the cells, after enzymatic digestion, pulp segments were minced and cultured (4–8 segments were fixed under a cover slip). Despite the control group, in diseased group red blood cells were found within 2–3 days after initial plating (Figure 1A). Single cell-derived colonies with heterogeneous sprouted cells emerged on day 3 to 4 in the control group and on day 6 to 7 in the diseased group. Interestingly, although the mean age of donors in the diseased group was 30 years old, 4 samples in the diseased group did not have any cell. The accumulation of tissue debris around the cells was noticeable. However, sprouted cells pushed debris via a negative chemotaxis response. This pattern seemed to be confined to the diseased group. The debris did not have the chance to disrupt the process of cell division (Figure 1D).

Furthermore, DPSCs were able to differentiate into osteogenic (Figure 2A and 2B) and chondrogenic (Figures 3A and 3B) lineages. hDPSCs were also tested with flow cytometry for the presence or absence of characteristic markers. MSCs typically expressed the antigens CD105 and CD73. Furthermore, cells expressed CD90 (Figures 4A, 4B, and 4C). They were negative for the early hematopoietic marker CD34 (Figure 5).

We sought to examine the expression of Nucleostemin, Oct-4, jmj2c and Nanog as proliferation and self-renewal regulatory

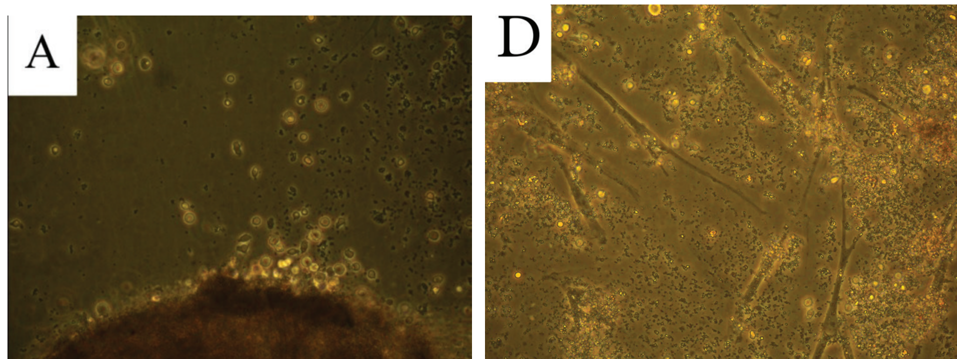

Figure 1. A) Part of the tooth pulp (×200). Red blood cells were exiting from the pulp. **D)** Stem cells, was surrounded by too much tissue's debris. Stem cells removed debris by negative chemotoxicity. After one week of growing cells, which were exiting from pulp were attaching on the flask surface and slide surface during passage time. It was noticeable that both flask surface and slide surface next to the pulp need to be removed carefully.

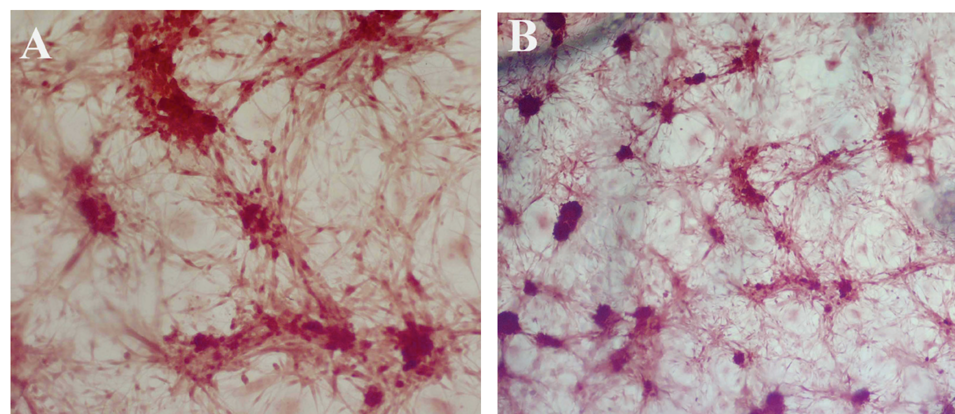

Figure 2. The human DPSCs were able to differentiate toward osteogenic lineage (A: ×200) (B: ×100).

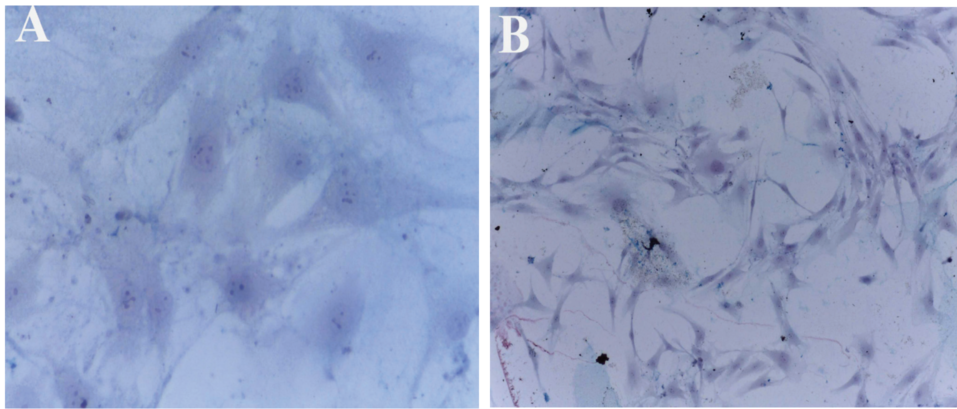

Figure 3. The human DPSCs were able to differentiate toward chondrogenic lineage; staining With Alizarin blue (A: ×400) (B: ×100).

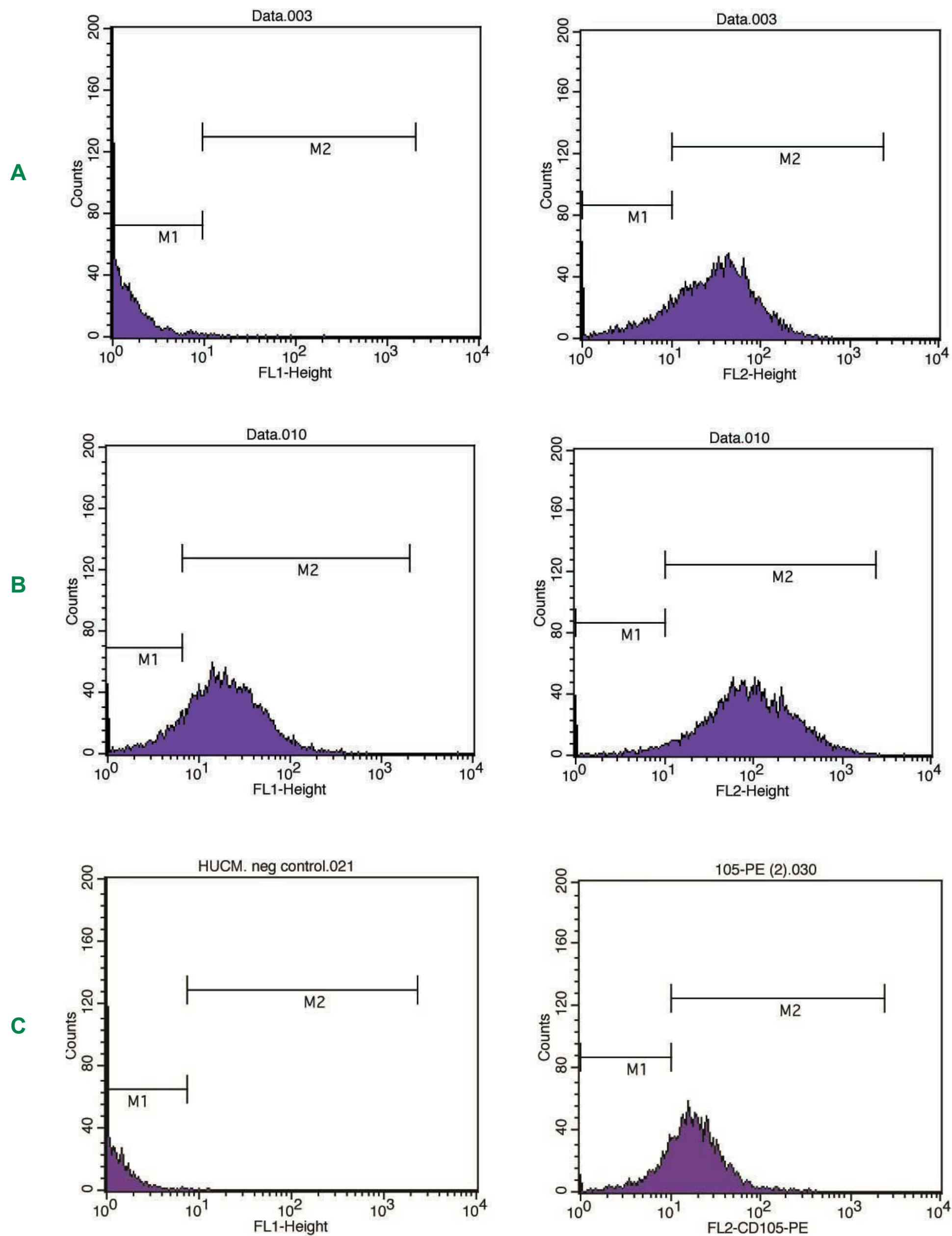

Figure 4. The human DPSCs from teeth with advanced periodontal disease showed high expression of surface markers A) CD73, B) CD90, and C) CD 105.

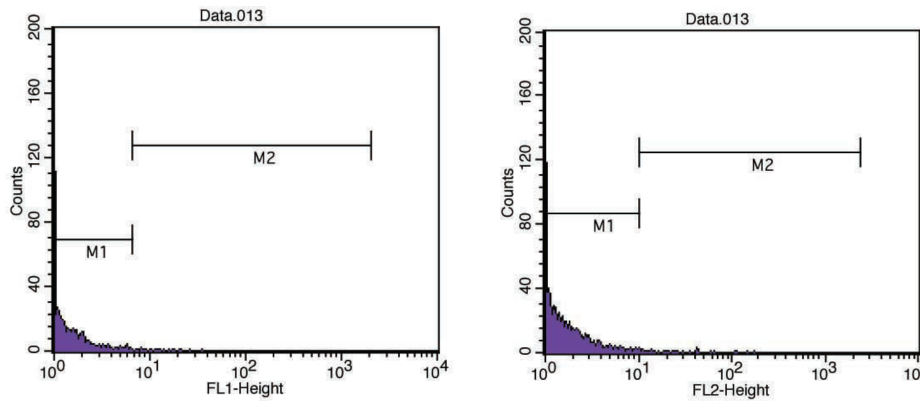

Figure 5. The human DPSCs from teeth with advanced periodontal disease showed no expression of CD34.

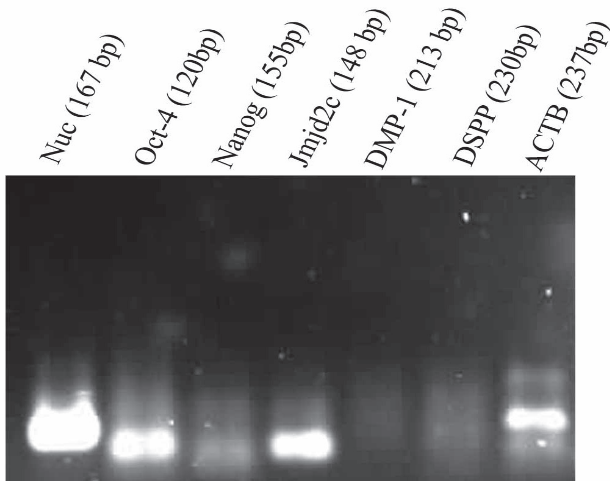

Figure 6. Agrose Electrophoresis Gel (RT-PC), ACTB gens, NS, Oct4, Nanog, Jmj2cs were grown in Human's pulp stem cells with perio problem. After 18 days during third passage, it was shown that ACTB genes (control), NS, Oct4, and Jmj2c had a high expression and Nanog had a lower expression; and no adentoblastic expression was seen in those cells.

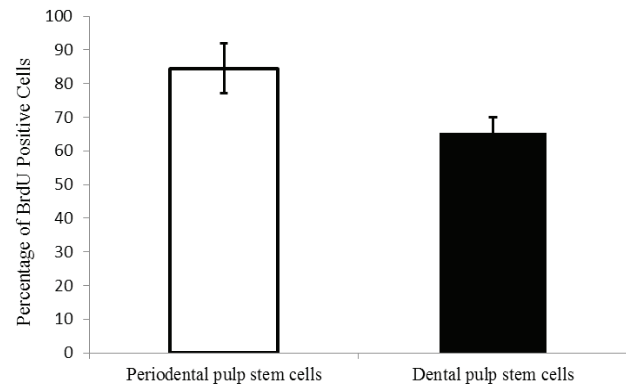

Figure 7. In each group, five randomly selected areas of BrdU-positive cells were found in each of the five regional averages were calculated for comparison with other groups; $P = 0.001$; CI = 95%, *t-Test*.

factors for mesenchymal stem cells. Based on the RT-PCR and agarose gel electrophoresis followed by Image Lab software (Bio-Rad, USA) quantitative analysis, nucleostemin, Oct-4 and jmj2c, but not Nanog, were expressed in undifferentiated mesenchymal stem cells of both groups. In addition, the Nucleostemin gene was expressed much higher than the others (Figure 6). Interestingly, diseased pulp exhibited higher gene expressions although it was not statistically significant. However, markers specific for odontoblast cells such as DSPP and DMP-1 showed no expression (Figure 6).

The average percentage of BrdU positive cells in the diseased group (84.4%, n = 5) was significantly higher than that of the control group (65.4%, n = 5) (*t*-test, $P = 0.001$) (Figure 7). It shows that diseased pulp cells exhibit a higher proliferation potential than healthy pulp cells.

Discussion

Hopeless periodontally involved teeth are believed to have no value and are therefore discarded after extraction. However, our study showed that there are some viable cells in the pulp of these teeth with the potential of *ex vivo* expansion and proliferation. Moreover, hDPSCs acquisition from this source has an extra advantage as stem cells can be obtained through a non-invasive surgical procedure. Limited information is available on the existence of functional DPSCs in such clinically compromised teeth. Our findings are in line with a previous study showing that teeth with complicated crown fracture have functional DPSCs.[16] Also, a study by Wang *et al*. demonstrated that there are putative stem cells in human dental pulp with irreversible pulpitis.[17] Chen *et al*. indicated that hDPSC cultures could be established by isolating cryopreserved dental pulp tissues from diseased but vital teeth, using a digestion and culture procedure after thawing.[18]

It has been shown that the isolation method has a bright effect on population expansion.[19,20] Two methods have been used extensively to culture dental pulp stem cells. The first is the enzyme-digestion method and the second is the explant outgrowth method.[19] Recently, Raoof *et al*. proposed a new approach with high efficacy to obtain dental pulp stem cells in a short time.[20] They digested the pulp tissue pieces and fixed them. This novel method provided the greatest *in vitro* expansion compared to

the two mentioned above. Moreover, the cells were less damaged and were therefore healthy enough to propagate longer *in vitro* than the other methods. So, we used this approach including digestion of pulp tissue pieces and fixing them for our study.

It is not clear why cells from some diseased samples did not form colony in primary culture. However, we were able to isolate dental pulp stem cells from donors aged 35–40 years old. Therefore, age may not be a major limiting factor for dental pulp stem cell isolation. This finding is consistent with data from a study by Chen *et al*. suggesting that there are no differences between hDPDCs derived from young or old patients in terms of growth rate and stem cell features such as the ability to undergo chondrogenic, osteogenic, and adipogenic differentiation.[21]

Our findings also showed that the pulp stem cell isolation efficiency in molars was much higher than anterior teeth. Each dental placode is suggested to give rise to an entire tooth family (incisor, canine, and molar).[21] In this regard, they may have somewhat different characteristics.

There were remarkable masses of debris around the cultured tissue that was limited to the diseased group. However, the debris did not affect the proliferation rate *in vitro* and the diseased group showed slightly faster separation of Mesenchymal stem cells. It seems that negative chemotaxis is involved in this successful process. After a period of time, primary cultures reached confluency and the debris disappeared. After the passage, the cells continued to grow with competent proliferation capacity.

To characterize the expression of various markers implicated in the self-renewal capacity of mesenchymal stem cells, we performed an evaluation of expression of Nanog, Oct-4, Jmjd2c, and nucleostemin[22] by RT-PCRWe used a established protocol for separation of mesenchymal stem cells from dental pulp cells on the basis of their adherence properties. Probably, cells isolated by this method constitute a heterogeneous population and therefore, we cannot exclude the possibility that Nanog is only expressed in a small subpopulation making overall expression at levels too low for reliable detection by routine RT-PCR. However, expression of nucleostemin, Oct-4, and Jmjd2c genes suggests a potential role for these genes in regulating self-renewal and the differentiation potential of these mesenchymal stem cells. Therefore, DPSCs might be used as an autologous stem cell source for cellular therapy and tissue engineering applications.

Interestingly, proliferation of pulp cells in the diseased group was higher than the control group in expanded passages, indicating that proliferation potential is not only compromised, but is still robust. Besides, the expression of mesenchymal stem cell markers was also higher in the diseased group. It seems that mesenchymal stem cells derived from these hopeless teeth that are often extracted and discarded as contaminated waste, might be extremely promising in terms of regenerating tissues.

Additionally, Papaccio *et al*.[23] demonstrated that following storage in liquid N_2 for 2 years, hDPSCs were still capable of differentiation into bone tissues.[23] Therefore, banking freshly digested hDPSCs is clinically possible for future usage. Moreover, Perry *et al*.[24] reported that hDPSCs isolation is possible and practical even 120 h after tooth extraction. As a consequence, immediate isolation of hDPSCs for successful banking after tooth extraction might not be absolutely required. In this regard, dental practitioners have the opportunity to make their patients aware of these invaluable sources of mesenchymal stem cells that can be stored for future application, as new mesenchymal stem cell based therapies are developed for a range of diseases and injuries.

In conclusion, our exploratory study suggests successful isolation of mesenchymal stem cells from the pulp tissue of hopeless periodontally involved teeth. The data also revealed nucleostemin, Oct-4, Jmjd2c but not Nanog, as the plausible regulators of DPSCs replication and self-renewal. Further characterization of these cells will provide insight regarding whether they could serve as a source of endogenous multipotent cells in tissue regeneration based therapies.

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