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Neural Differentiation Potential of Stem Cells Derived from Dental Follicle and Periodontal Ligament stem cells

Naghmeh Bahrami[1], Gholamreza shirani[1*],Yadollah shayesteh soleymani[2*], Zeynab Manafi[3], Fatemeh Mohajeri[3] , Abdolreza Mohamadnia[4]

1- Craniomaxillofacial Research center, Tehran University of Medical Sciences, Tehran, Iran. Oral and Maxillofacial Surgery Department, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran

2- Dental Implant Research Center and Department of Periodontics, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran

3- School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran

4- Virology Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran. Department of Biotechnology, School of Advanced Technologies in Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

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Abstract:

Background: Repair of neural damages is one of important and complex treatment in medicine and dentistry. On one side possession of adult stem cells from bone marrow considering limited source of these cells, is restricted. Finding reachable source of stem cells with high differentiating potential is important. These sources include PDL and dental follicle. The goal of this research is to compare potential of neurogenic differentiation of stem cells derived from periodontal ligament (PDLSCs) and stem cells derived from dental follicle (DFSCs) of impacted third molar.

Methods and Materials: in this experimental study, 3 samples of PDL and 3 samples of impacted 3rd molar follicle were used to isolate stem cells. Cultivation of digested tissue pieces was used. Differentiation to osteoblast and adipocyte was used to prove the existence of stem cells. Also to prove the mesenchymal origin of stem cells, expression of mesenchymal cell surface markers CD44, CD73, CD90, CD105 and hematopoietic cell surface markers CD34 and CD45 were examined with flow cytometry. At the end, neural differentiation of cells cultured in standard neural inductive medium containing retinoic acid was investigated by expression of MAP2 and b-Tubulin genes and their protein products by RT-PCR and Western blot technique.

Results: Stem cells were successfully isolated from dental follicle and PDL tissues. All cells were successfully differentiated to adipocyte and osteoblast after passing of 14 days in adipogenic and osteoblastic inductive mediums. Flowcytometry revealed that these cells are positive for CD44, CD73, CD90, and CD105 and negative for CD34 and CD45 and also expression of MAP2 and b-Tubulin were confirmed by RT-PCR and western blot tests.

Conclusion: Results showed that more neurogenic differentiating potential was detected in derived stem cell from PDL in compared to tooth follicle cells. This study demonstrated that dental follicle and PDL can be used in the cellular treatment and tissue engineering in neural damages repair.

Key Words: Dental follicle, PDL, Stem Cell, Neural Differentiation, Nerve Tissue Engineering.

*Corresponding Authors: Gholamreza shirani, Yadollah shayesteh soleymani
Tel/Fax: +989121098516, shirani@sina.tums.ac.ir,
Tel/Fax: +989125088015, Drshayesteh20@yahoo.com,

Introduction

One of the most common injuries during dental treatment is damage to nerves. During many common practices such as dental anesthetic injection, third molar surgery, surgical jaw lesions, the use of implants and etc there is the possibility of damage to the nerves. The vast majority of neurons are formed in embryonic stage but after birth do not have the ability to reproduce through mitosis. In this way there is no possibility of healing the neurons destroyed by disease, trauma or chemicals. Although neurons after birth are unable to reproduce, they can resist against to a certain degree of lesions and improve which is called regeneration process (1). Following nerve injury, nerve tries to repair itself by sprouting of nerve treatment units. The treatment units will then try to re-innervate the muscles and skin. If a correct connection occurs between nerve and muscle motor or sensory nerves and the skin, as a result of repair, recovery of muscle or skin sensation will happen. But if for any reason this connection is not restored to the correct shape, there won't be any recovery. After neurotmetic injuries, neuroma is created usually as the result of efforts of axons by random path for nerve regeneration (2).

Nerve regeneration in the treatment of nerve damage is a major target which is used in tissue engineering. In general, tissue engineering and its related researches in dentistry and medicine regeneration has a special position. Tissue engineering has three basic parts, including cells, scaffolds and growth factors. A key factor in integrating these technologies with modern practices is the use of adult mesencymal stem cells (MSC). Adult stem cells are pluripotent and can differentiate experimentally into different cell lineage (3).

Most stem cells that have been studied until now are mesencymal stem cells from blood or bone marrow. Due to the limited supply of bone marrow MSC were isolated, there needs to be an alternative source for MSC. In human postnatal dental tissue, five different sources of mesenchymal stem cells (MSCs) have been already identified: dental pulp, periodontal ligament, exfoliated deciduous teeth, dental follicle (DF), and root apical papilla (4).

Dental follicle is a loose connective tissue. Dental follicle stem cell (DFSCs) can be a source of MSCs (5). Dental follicle exists in impacted teeth. Usually these teeth are discarded during dental treatments.

The periodontal ligament is a specialized connective tissue. PDL serves a supportive function by attaching the tooth to the surrounding alveolar bone properly. This function is mediated primarily by the principal fibers of the periodontal ligament that forms a strong fibrous union between the root cementum and the bone (6). Dental PDL containing progenitor cells. This texture can be separated easily from impacted and semi-impacted teeth (7).

PDL and dental follicle stem cells can be isolated and grow in defined tissue culture conditions and the final properties of these cells, increases their potential for use in tissue engineering approaches, including neural and bone tissue regeneration.

Material and Methods

Isolation and primary culture of DF&PDLSC stem cells

Impacted wisdom teeth used in this study were obtained from 6 patients after surgery. The ethics committee code was (IR.Tums.REC.1394.1438). Approval and written informed consent was obtained.

The mean age of the patients enrolled was 15-30, and they were free of any disease and didn't smoke or use alcohol. After identifying dental surgery, the place of surgery was washed with chlorhexidine solution 0/2%, lip and skin of the patients were scrubed by betadine diluted. Wisdom tooth extraction was performed under local anesthesia. After lifting the flap completely, maxillary or mandibular bone tissue on the teeth was removed due to burrs 029-023 and under irrigation with sterile normal saline to prevent any damage for adjacent tissue. Available follicle tissue isolated in the crown and PDL isolated from root into the container containing the liquid hanks moved quickly to the laboratory. In the laboratory they were washed several times under the hood, using sample buffer PBS (Phosphate Buffer Saline) containing antibiotics. Then they solved in a solution of solvent tissue containing tissue culture medium, DMEM (Dulbeccos Modified Eagle Medium) and digested with collagenase type 1 with a concentration of 250 u/ml for 1 to 2 hours in a shaking incubator at 37 ° C and CO2 5%. After digesting the tissue, to remove undigested pieces of tissue and impurities, filtration was conducted by filters 70 and 40. Ficol was used to remove mononuclear cells. The solution containing the sample was centrifuged into centrifuges (eppendrof centrifuge 5810R) whit around 1500 rpm for 5 minutes to form a cell plate. After that, the cells were cultured in the flasks T75 containing DMEM and FCS 10%.After 48 hours, the culture was replaced and then the medium was replaced every 72 hours. When 80% of the flask was covered by cells, they were passaged at a ratio of 1:3, using trypsin 0/2% and EDTA. To prove that the cells are stem ones, their differentiation into osteoblasts and adipocytes was used. Cells were derived mesenchymal stem cells of mesenchymal markers C44, CD73, CD90, CD105 and hematopoietic markers CD 45 and CD34 by flow cytometry.

Neural differentiation

Four groups were studied: group1. DFSCs in normal culture medium (only DMEM+FBS 10% and no differentiating factor added into medium), group2. DFSCs in standard neural inductive medium (8) (Dexamethasone 10nM + Ascorbic acid 50 µgr/ml + Retinoic acid phosphate 10mMol), group3.PDLSCs in normal culture medium and group4.PDLSCs in standard neural inductive medium. After passing of 14 days, cell differentiation was investigated by morphology, neuron specific gene expression (MAP2 and b-Tubulin) and western blot.

Morphology

Cells cultured in different groups were morphologically studied by an optical microscope.

Quantitative real time PCR (RT-qPCR)

RT-PCR was performed to analyze and confirm the differentiation of PDLSCs and DFSCs into neural cells. In this analysis, the expression of MAP2 gene and b-Tubulin gene was analyzed as neuron-specific genes. Moreover, GAPDH gene expression was considered “the positive control” in all cells. The primers used were asfollows:

Table 1. Primers used for real time RT-PCR.

Gene	Length(bp)	Primer sequence(50-30)	Annealing (_C)
GAPDH	15 20	F: AAA TTG AGC CCG CAG CCT R: GGGTTGAGCACAGGGTACTTTA	60
B-Tubulin	21 20	F: GGAGGGGCATCTCTTGAGAAC R: TCGAGGCACGTACTTGTGAG	60
MAP2	20 20	F: GCTCCCGGAGAAGGATTCTG R: TCAGGTGGATGTGAGTGTGC	60

According to the manufacturer's recommendations, all RNA was extracted, using AccuPower® RocketScript™ RT PreMix kit from Bioneer. The cDNA preparation process was continuously carried out in a thermocycler.

Western Blott

To confirm protein expression following neuronal induction, we harvested human DFSCs and PDLSCs grown under control conditions after neuronal induction for Western blot analysis. We probed the blots with primary antibodies to human MAP2, beta tubulin and beta actin. Human NT2 served as a positive control for MAP2 AND beta tubulin.

Results

Isolation and cultivation of DFSCs and PDLSCs

DFSCs and PDLSCs were successfully isolated from dental follicle and PDL tissues. To evaluate proliferation abilities, passage 3 PDLSCs and DFSCs were plated at 1000 cells/well and cultured for 1, 3, 5 and 7 days with optimal culture medium. Cells were morphologically investigated at 1, 3, 5 and 7 days after culture. On the third day, cell colonies were observed in culture medium (Figure1, Figure2).

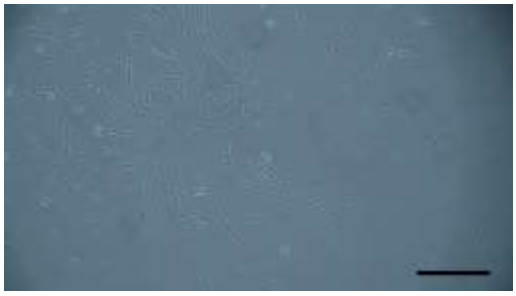

Figure1: PDLSCs in culture medium at day 3 (Scale bar: 100 um)

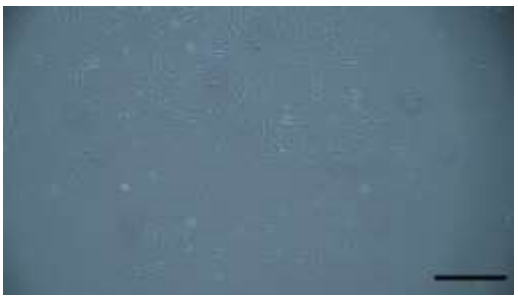

Figure2: DFSCs in culture medium at day 3 (Scale bar: 100 um)

Stem cells differentiation into adipocyte and osteoblast

After 14 days, all cells were successfully differentiated into adipocyte and osteoblast. When placed in an osteogenic differentiation medium, osteoblasts formed and developed mineralization nodules. Formation of mineralized nodules and calcium deposite was proven by Alizarin Red staining.(Figure4.A ,Figure4.B) When cells grown in stem cell medium were placed in an adipogenic medium and then stained with Oil Red O, stained adipocytes were observed, showing the production of lipid drops inside adipocytes (Figure3.A ,Figure3.B).

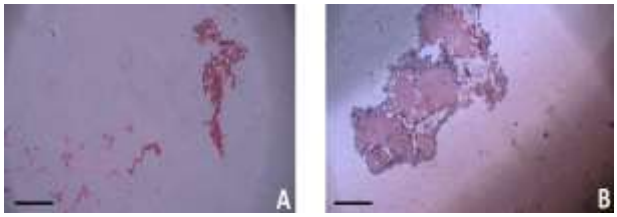

Figure3: In vitro lineage differentiation potential of DFSCs (A) and PDLSCs (B). Adipogenesis was detected by Oil red O staining of lipid droplets. (Scale bar: 100 um)

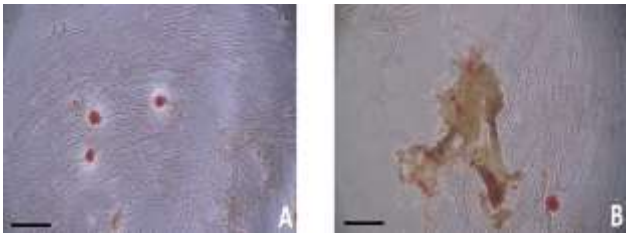

Figure4: In vitro lineage differentiation potential of DFSCs (A) and PDLSCs (B). Osteogenesis was demonstrated by Alizarin red staining of mineralized nodules and calcium deposition. (Scale bar: 100 um)

Flow Cytometry

DFSCs and PDLSCs after passage 3 were evaluated for expression of cell surface markers. Both cell types were positive for CD90, CD105, CD73, and CD44 cell markers, which are specifically related to mesenchymal stem cells; the cells were however, negative for CD45 and CD34. The expression of cell surface markers in DFSCs were %99.2 for CD44, %99.6 for CD73, %99.8 for CD90, %97.6 for CD105, %0.587 for CD45 and %0.845 for CD34. In PDLSCs were %97.4 for CD44, %99.3 for CD73, %100 for CD90, %96.6 for CD105, %1.04 for CD45 and %0.715 for CD34 (Figure5, Figure6).

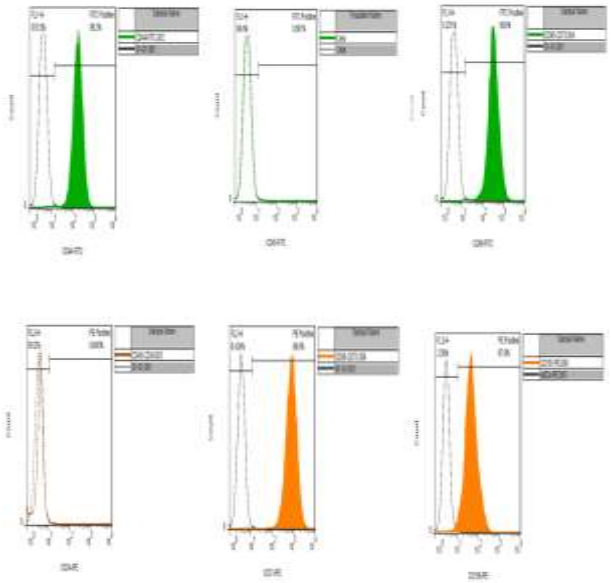

Figure5: Diagram of flowcytometery with isotype control sample in DFSCs

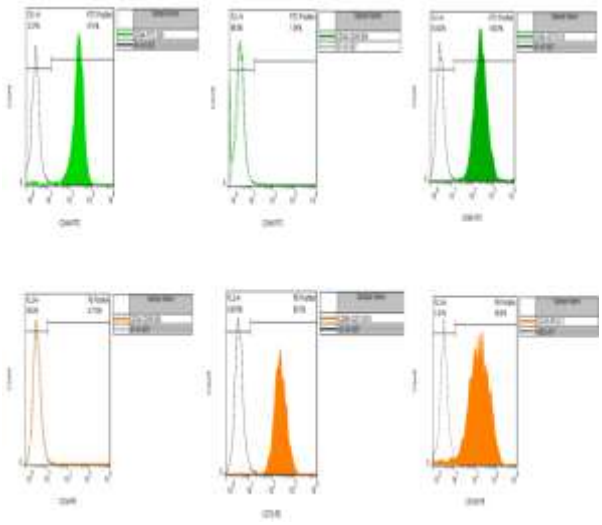

Figure6: Diagram of flowcytometery with isotype control sample in PDLSCs

Morphology

Morphology of cells after differentiation in group of neural induction medium containing retinoic acid and group of normal culture medium without retinoic acid was studied. As it can be seen, in cells of normal culture group without retinoic acid no differentiation happened and no neural cell morphology occured. In group of standard neural cells, they were differentiated into neurons and production of neural cells elongations were seen in microscope (Figure7 A, B , C, D).

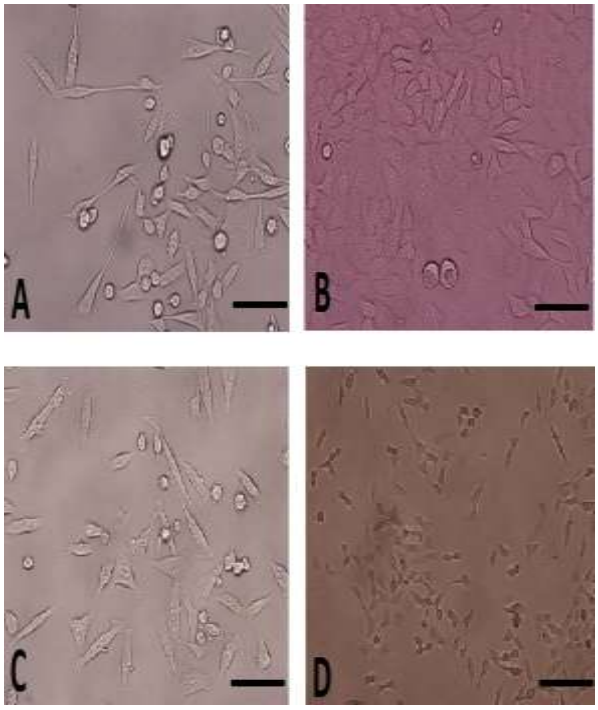

Figure 7: Phase contrast microscope picture after 14 days. A: DFSCs in standard neural inductive medium containing retinoic acid. B: DFSCs in normal culture medium without retinoic acid. C: PDLSCs in standard neural inductive medium containing retinoic acid. D: PDLSCs in normal culture medium without retinoic acid. (Scale bar: 100 um)

Expression of MAP2 and b-Tubulin on mRNA level

Considering expected results of constructed primers (MAP2=329bp) and (b-Tubulin=273bp), the results of the experiment were confirmed by DNA ladder 100bp. Group of negative control (1st column) containing water showed there was no RNA and DNA contamination in reactory materials. The expression of these genes in normal culture group without existence of differentiating factors (2nd column) was not significant, in other words, cells of these groups didn't express MAP2 and b-Tubulin genes. In standard neural cells along with differentiated factors (3rd column) MAP2 and b-Tubulin genes were explained. Positive control group (4th column) shows expression of these genes in NT2 cells (Figure8).

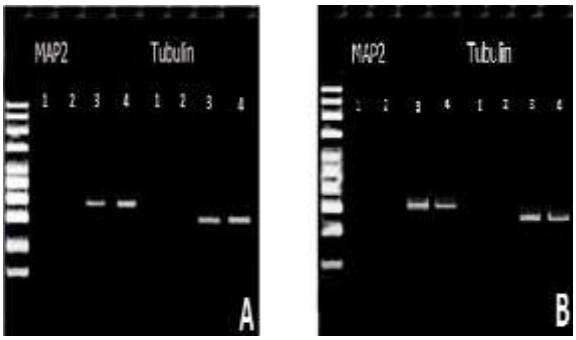

Figure 8: Real-time PCR analysis of DFSCs and PDLSCs. Agarose gel Electrophoresis for RT-PCR production of MAP2 and b-Tubulin genes in stem cells derived from 3th molar follicle (A) and PDL (B) after 14 days of cultivation in different groups in DNA ladder fermentase 100bp. Group1: negative control (H2O), Group2: standard neural inductive medium containing retinoic acid, Group3: normal culture medium without retinoic acid, Group4:NT2.

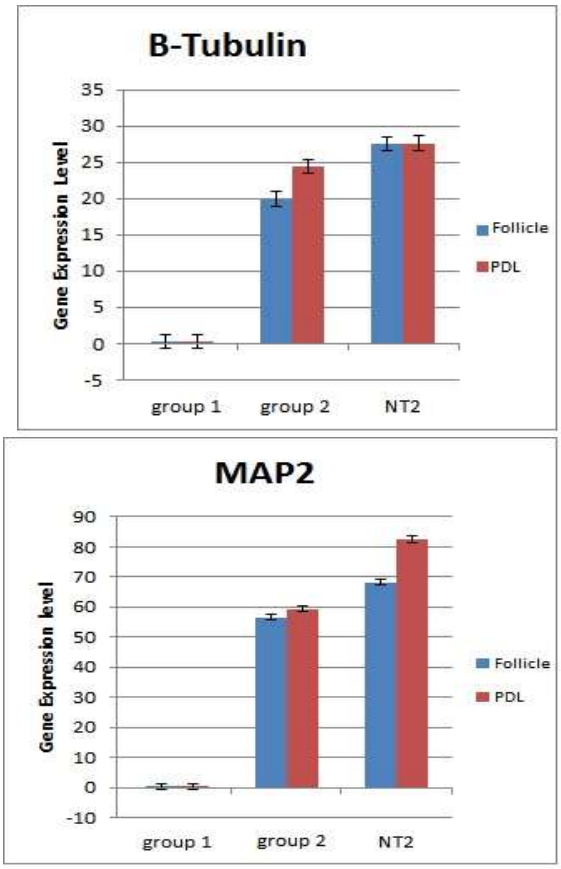

Figure 9: Real-time PCR comparison of MAP2 and b-Tubulin gene-expression levels in DFSCs and PDLSCs.

Expression of b-tubulin and MAP2 on protein level with western blot technique

Gene expression on protein production level with western blot technique was examined. Results of the experiment were consistent with RT-PCR results. Groups investigated were as followed:

1. Normal culture medium without existence of differentiating factors.
2. Standard neural inductive medium with differentiating factors.
3. Positive control group containing NT2 cells.

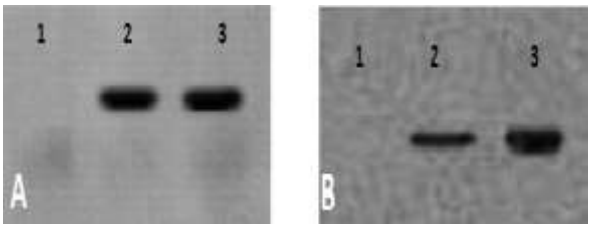

Figure 10: Expression of b-Tubulin protein (A) and MAP2protein (B) in DFSCs after 21 dayes culture in different mediums.1: Normal culture medium without differentiating factors, 2:2.Standard neural inductive medium with differentiating factors, 3:NT2

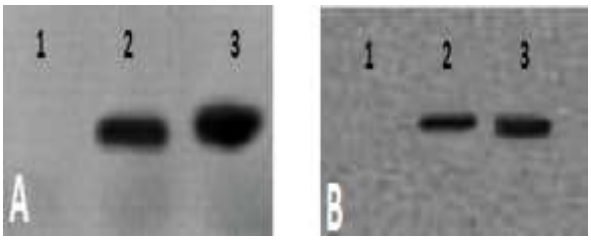

Figure 11: Expression of b-Tubulin protein (A) and MAP2protein (B) in PDLSCs after 21 dayes culture in different mediums.1: Normal culture medium without differentiating factors, 2: 2.Standard neural inductive medium with differentiating factors, 3:NT2

Discussion

Treatment of neural lesions and injuries are quite complex today. Tissue engineering based on cell therapy is well considered in regenerative medicine. Neural tissue engineering is the best choice for treatment of neural injuries.

In the present study, stem cells from PDL and follicle of impact third molar were used. In order to prove that stem cells are mesenchymal, cell surface markers were revealed by flowcytometery analysis. High Expression of CD44, CD90, CD73 and CD105 and

low percentage near zero of CD34 and CD45 markers showed that cells didn't have endothelial and hematopoitic origin. In other researches, other cell surface markers were examined to prove the existence of stem cells in the cell populations by Immunocytochemistry and flowcytometry such as CD146, CD166, HLA-1, sox2, klf4, nanog and c-myc (9-11). Then samples were seed in standard medium of osteogenic and adipogenic inductive medium and according to 14 days protocol of differentiation, they were differentiated into osteoblast and adipocyte and by this mean they proved to be stem cells. Finally it was proved that PDL and tooth follicle tissue used in the present study had mesenchymal stem cells and these cells show they had potential of self-renewal to passage3 because they differentiated into osteoblast and adipocyte. These results in addition to their capability of differentioation into specialized lineage showed possibility of usage in tissue engineering under favorable condition for differentiation.

Some studies such as ME Yalvac (11) and S Yao (12) used neurogenic differentiation in addition to adipogenic and osteoblastic differentiation to prove that isolated cells are stem cells. In a number of similar studies as well as the present one, only osteogenic and adipogenic differentiation has been done (13-15).

Mesenchymal stem cells considering they are originated from or going to which part of body, show different potential and differentiation power (16). The results were obtained from this study demonstrate that stem cells derived from PDL showed more neurogenic differentiating potential comparing to cells of tooth follicle. Rate of gene expression and neural cell protein in stem cells of PDL according to RT-PCR test and western blot were more than stem cells of 3th molar.

Regenerative potential of periodontal ligament-derived stem cells (PDLSCs) and osteoblast differentiated from PDLSC were compared with bone marrow-derived mesenchymal stem cells (BM-MSCs) and pre-osteoblasts in calvarial defects. The findings indicated that although PDLSCs and pre-osteoblasts could be used for bone regeneration, the rate of regeneration in BM-MSCs-treated cavities was more significant (17).

Stem cells isolated from dental pulp, follicle and PDL and their potential of neurogenic differentiation were compared. Study showed the three types of dental MSCs broadly possessed similar cellular properties and can differentiate into neuronal cells; however, pulp derived MSCs showed higher neurogenic potential than the follicle and papilla. According to results of present study, potential of neurogenic differentiation of PDLSCs was more than DFSCs. Results of this study proved that PDL and follicle are containing multipotent stem cells that have differentiating potency specially neural cells. These tissues are easily reachable and usually are thrown away during impact third molar surgery and stay unused (18).

Postnatal stem cells from human dental tissues such as dental pulp (DPSC), periodontal ligament (PDLSC), periapical follicle (PAFSC), and the surrounding mandibular bone marrow (MBMSC) were isolated to ascertain their properties (11).

Similar to present study, the isolated cells showed self-renewal capabilities and colony-forming efficiency. Almost all of the dental stem cells showed optimal growth when they were cultured in alpha

modification of Eagle's medium (alpha-MEM) supplemented with 10% fetal calf serum (FCS) and 100 lM ascorbic acid. Similar to Kadkhoda's study, MBMSC, in particular, showed much better mineralization compared to the others. These results indicate that MSCs exist in various tissues of the teeth and can differentiate into osteoblasts, adipocytes, and other kinds of cells with varying efficiency. This study, introduces dental follicle and PDL as available source of stem cells to be used in cellular treatment and tissue engineering especially in repair of neural damages. However, for the treatment of neurological using this Stem cell research needs to be wider.

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