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Comparison the differentiate ability of wisdom tooth follicle stem cells into nerve and bone tissue

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Abstract:

Background: The ultimate goal of craniomaxillofacial treatment is to achieve bone, nerve, cartilage regeneration with the reconstruction of all the components. The goal of this study was to assess Comparison the differentiate ability of wisdom tooth follicle stem cells into nerve and bone tissue

Methods and Materials: in this experimental study, 3 samples of impacted 3rd molar follicle were used to isolate stem cells. After approved the mesenchymal stem cells, neural differentiation and bone differentiation of cells cultured in standard medium culture for neural differentiation and bone differentiation. Twenty-one days later the differentiation of the stem cells were examined and compared.

Results: Upon examining the gene expression of osteoblasts, osteocalcin, beta Tubulin genes and MAP2 the cultured specimens had statistically desirable osteoblastic and neuroblastic induction.

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

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

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Introduction

The goal of tissue engineering is replacing damaged and diseased tissues or restoring functions of these tissues through the application of engineering and biological principles. These principles include the selection and application of cells, scaffold design and the identification and use of biological signaling molecules. It is expected that texture produced by this method is structurally and functionally similar to the target tissue. Currently, there are many works in progress to study and use tissue engineering in dental treatment (1).

Stem cells are a class of undifferentiated cells that have self-renewal properties and have the capacity to differentiate and turn into the other cell. Today, stem cells are the first hope to repair damaged tissues and perhaps in the future making human organs would be possible this way (2).

According to their origins, stem cells are divided into two categories: embryonic stem cells that are taken from embryos in early stages of its formation, and mesenchymal or adult stem cells that are taken after the birth of people, especially from their bone marrow (2,3). Given the incidence of some restrictions on the use of embryonic stem cells including moral constraints on the supply of these cells, as well as the difficulty of controlling the growth and proliferation of these cells due to the high growth potential and their tumor genesis, a new wave of research started mesenchymal stem cells that continue (4-6).

In the past decade, many studies have reported the separation of stem cell population from different dental resources including dental pulp, periodontal ligament, missing milk tooth, the dental follicle, and root apical papilla (7).

Here, wisdom tooth follicle is a readily available source of stem cells that can easily be extracted from impacted wisdom teeth removed with surgical procedures. In humans, the formation of the third molar structure is unique because the process of organogenesis begins after birth and at the age of 6 and up to this time, embryonic tissues remain quiet and undifferentiated in the dental tissue. (8)

Moreover, most of the skeletal and dental problems happen in adulthood which baby teeth are absent. Since the third molars tooth is pulled for routine prophylactic or orthodontic reasons and remains useless, the isolation of mesenchymal stem cells from tissues of the third molars tooth is a good option (9,10).

In the past, scientists believed that adults' stem cells are able to create only cells of the same texture, but today they believe that these cells can also be converted into other types of cells (11,12). Directed differentiation of stem cells is vital for their use in developing new treatments (13,14).

The bone that provides mechanical support for the creature is a place for blood production and a storing and homeostasis of calcium ions. Therefore, it is necessary to maintain its health for survival (15). Osteoblast cells play an important role in bone formation (16). Mesenchymal stem cells are among cells that play an important role in bone tissue regeneration and damaged cells restoration. In addition, in most studies, the differentiation of mesenchymal cells into bone is taken into consideration as a part of the potential (14).

As we know, the nerve cells are incapable of cell division and proliferation after birth that is why in the nerves experiencing severe damage or injury caused

by the tumor and nerve severed, in the area a gap is created between the two areas, and there is a need for surgical intervention (17).

Massive improvements in surgical tools and techniques have improved the treatment of peripheral nerve injuries. However, constraints such as shortage of transplantable nervous, very difficult and long process of surgery, postoperative complications such as scarring, and pain in the area of wound infection and poor regeneration of nerve tissue make the process difficult.

Since in cell-therapy strategies, fully differentiated cells are used, the first step in using mesenchymal cells in order to reconstruct bone and nervous defects is their differentiation into osteoblasts and neurons in vitro (14).

The aim of this study is to use human wisdom tooth follicle stem cells for neural and bone tissue engineering for treatment of injuries birth defects and bone, especially in the jaw and face.

Material and Methods

Sampling method

In this experimental study, three healthy impacted wisdom teeth of patients in the age group 18-30 years were obtained with written and prior consent. Patients without infectious and systemic diseases without any history of drug use, smoking, and alcohol were not enrolled. The wisdom tooth of these patients needed surgery for orthodontic reasons or lack of growth. After identifying teeth for surgery, patients had mouthwash with chlorhexidine solution 0.2%, and lip and skin of his face were scrubbed using diluted iodine.

Wisdom tooth extraction was performed under local anesthesia. After fully lifting of the flap, maxillary or mandibular bone tissue due to the teeth was removed with burrs 029-023 under irrigation with sterile normal saline to prevent any damage to adjacent tissue. After removing the tooth, tooth follicle tissue was separated by scalpel into hanks liquid and was quickly transported to the laboratory.

Isolation and culture of stem cell

In the laboratory in sterile conditions under the hood, wisdom tooth follicle sample was rinsed for several times by Phosphate Buffer Saline (PBS) containing antibiotics. Then the tissue was placed in a solution of Dulbecco's Modified Eagle Medium (DMEM) and collagenase enzyme type I with a concentration of u/ml 250 for 1 to 2 hours in a shaking incubator at 37 ° C and the CO2% 5 for tissue digestion. After digesting the tissue, to remove undigested tissue fragments and impurities, filtration was done by 70µm and 40µm filters.

Ficole was used to remove mononuclear cells. A solution containing the sample was centrifuged in a centrifuge (Eppendorf centrifuge 5810R) at 1500 rpm for 5 minutes to form cell plate. After that, the cells were cultured in T75 flasks containing DMEM and FCS 10% medium. After 48 hours, the culture was changed and then the culture medium was replaced every 3 days. When 80% of the flask was covered by cells, the cells were separated by trypsin and EDTA from the flask and were passaged at the ratio of 1:3. To prove cell being stem cells, differentiation of osteoblasts and adipocytes was used.

Differentiation of stem cells of wisdom tooth follicle into osteoblasts and adipocytes

To prove the cells being stem cells, using the medium of differentiation, we differentiated them into fat and bone. After the third passage, the cells were prepared

to study differentiation into fat and bone cells. To study the differentiation into fat and bone cells, 20000 cell/ml were cultured in a 24-well plate containing 12 DMEM / F and 10% in serum. After 24 hours, the medium of differentiating into fat and bone was added to the cells. Adipocytes differentiation medium consisted of DMEM/F12 with 50 micrograms per ml ascorbic acid.

The cells were incubated for 14 days in the induction media and cell media was replaced three times per week. In order to show areas differentiated into fat cells, Oil Red staining was used. To stain with Oil red, cells were fixed in 4% formaldehyde for 60 minutes and were washed with 70% ethanol. Then the stain was used that was composed of three parts of stock solution (0.5% Oil Red O in isopropyl alcohol 99%) and two parts of distilled water. To demonstrate differentiation of mesenchymal stem cells into bone cells, Alizarin Red staining was used.

Alizarin Red is an organic compound that specifically stains the mineral cells matrix in the red color so that the intensity of staining of the tissue is directly related to a number of minerals in the matrix.

Flow cytometry

In this study, flow cytometry analysis was used for studying phenotypic profiling of surface markers and the nature of follicle stem cells extracted from human third molars tooth. After the third passage, the markers CD 105, CD90 and CD44 were used as markers of mesenchymal cells, and CD34 and CD45 as a marker of and hematopoietic cells. Flow cytometry of antigen of specific mesenchymal cells was as follows (it should be noted that these antigens are cell surface antigen components):

In the third passage, cells were trypsinized and in form of suspension as many as 10^5 in a volume of 100 ml of (Phosphate Buffer Saline) PBS were transferred to separate tubes. Then they were added to CD44-FITC (Exbio / Czech), CD90-FITC (Exbio / Czech), CD45-FITC (Exbio / Czech), CD73-PE (Exbio / Czech), CD34-PE (Exbio / Czech), and CD105-PE (Exbio / Czech) antibodies as much as 5 µl.

At the same time, in separate tubes, Mouse isotope control antibodies IgG1-FITC, Mouse IgG1-PE Mouse IgG2a-PE were added to the same cell number. Then these tubes were incubated at 4 ° C for 30 minutes in a dark environment (Dark room). After this period, the cells were washed with 1ml washing buffer for 5 minutes in a centrifuge with 1500RPM speed. Then each cell was a suspension in 250µl washing buffer and immediately was read for FITC and 585 / 42BP by flow cytometry BDFACS Calibur (BD bioscience USA, CA, Sanjose) with 530 / 30BP Filters. The data obtained were analyzed in the Flowjo.7.6.1 software.

Osteogenic differentiation induction to DFSCs

In order to induce bone differentiation to DFSCs, the cells were cultured in 2 separate containers. The first container as a negative control contained only normal culture medium (DMEM + FBS 10%) and no differentiation factor was added to it. the second container, DFSCs were placed in standard osteogenic medium (Dexamethasone 10ng + Ascorbic acid 50µg / ml + Betaglycerol phosphate 10mMol). After 14 days, cells differentiation in morphology verification procedures, verification of gene expression in osteoblasts (osteoblasten and osteocalcin) by RT-PCR, Alizarin Red staining, immunocytochemistry,

and assessment of alkaline phosphatase were investigated.

Neurogenic differentiation induction to DFSCs

In order to induce neuronal differentiation to DFSCs, the cells were cultured in 2 separate containers. The first container as a negative control somewhere just the normal culture medium (DMEM + FBS 10%) and no differentiation factor was added to it. In the second container, DFSCs were placed in standard neurological cultivation (21) (Retinoic acid phosphate 10 mMol + Dexamethasone 10ng + Ascorbic acid 50µg / ml). After 21 days, cell differentiation was assessed by morphology approval procedures, approval of gene expression in neuronal cells (beta Tubulin genes and MAP2).

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To confirm protein expression following neuronal induction, we probed the blots with primary antibodies to human MAP2, beta tubulin, and beta-actin. And for bone induction, we probed the blots with primary antibodies to osteoblasten and osteocalcin.

Results

The results of isolation and culture of DFSCs

Stem cells were isolated successfully from wisdom tooth follicle tissue. Then the cell sample was placed in culture medium and at intervals of 1, 3, 5, and 7 days was evaluated for their morphology (Figure1). On the third day, cell colonies were observed in culture.

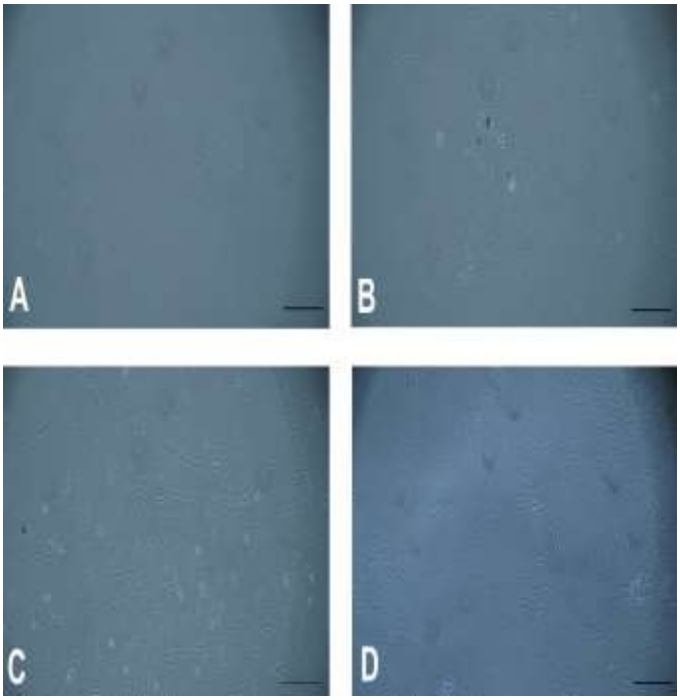

Figure1: DFSCs in the culture medium. A: day 1. B: day 3. C: day 5. D: day 7 (scale bar:100 um)

Differentiation of stem cells into adipocytes and osteoblast

To prove the potential of differentiation and pluripotency of the cells, DFSCs were placed in adipogenesis and osteogenesis. After 14 days, all cells differentiated into adipocytes and osteoblasts successfully. Osteogenic differentiation was proved by Oil red staining of intracellular lipid droplets in adipocytes. Osteogenic differentiation was proved by mineralized nodule formation and calcium deposition identified by Alizarin red staining (Figure2).

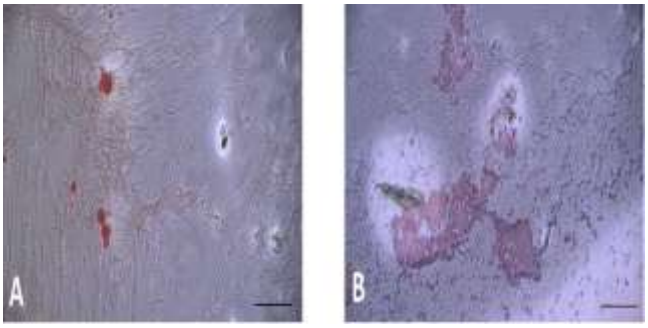

Figure2: In vitro lineage differentiation potential of DFSCs (A) Osteogenesis was demonstrated by Alizarin red staining of mineralized nodules and calcium deposition (B) Adipogenesis was detected by Oil red staining of lipid droplets (scale bar: 100 um)

The results of flow cytometry analysis

Flow cytometry results showed that after the third passage, these cells were positive relative to the mesenchymal marker CD44, CD73, CD90, and CD105, negative in relation to hematopoietic markers CD34 and CD45. Expression percentage of these markers in the dental follicle stem cells was 99.2% for CD44, 0.587% for CD45, 99.8% for CD90, 99.6% for CD73, 0.845% for CD34, and 97.6% for CD105(Figure 3).

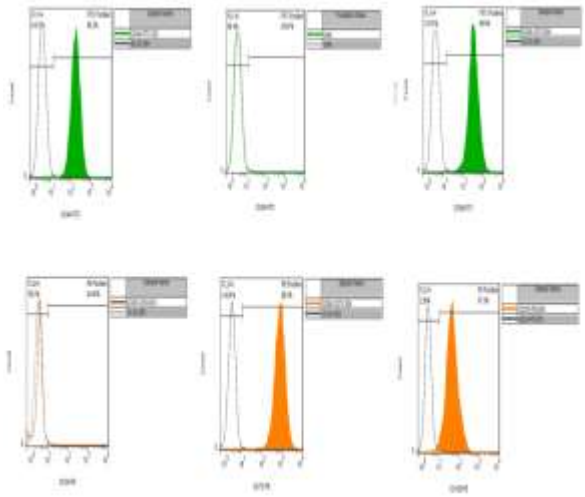

Figure 3: Histogram ISO type control flow cytometry with samples of wisdom tooth follicle cells

The results of bone morphology study

After 14 days, standard medium osteogenic cells and normal culture medium without BMP2 were examined for morphology light microscopy. In standard osteogenic medium containing a BMP2 medium, cells multiplied and so-called got hand into hand, but cells in medium without BMP2 gathered in different areas and went to die (Figure 4).

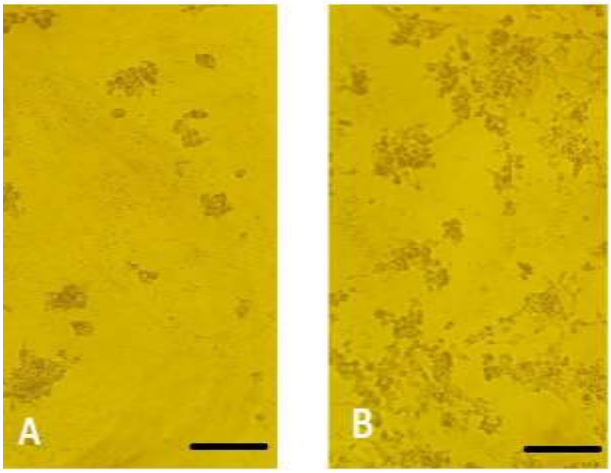

Figure 4: Phase contrast microscope picture after 14 days. A: DFSCs in normal culture medium without BMP2.B: DFSCs in the standard osteogenic inductive medium. (Scale bar: 100 um)

The results of studying neural morphology

Neural morphology of cells was examined after 21 days in standard normal culture medium and normal culture medium without morphology differentiation factor by light microscopy. As expected, in a normal cell culture medium without differentiation factor no distinction of any kind occurred and nerve cell

morphology happened. In a standard medium, neural cells differentiated into neurons and in microscope examination, nerve cell formation was observed (Figure5).

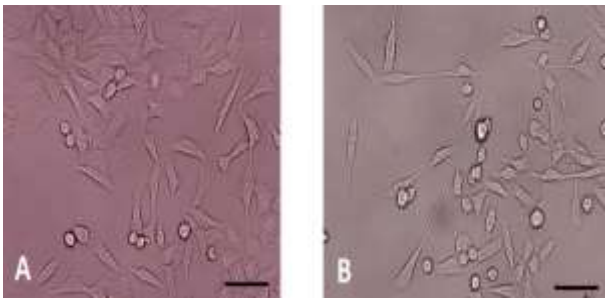

Figure5: Phase contrast microscope picture after 21 days. A:DFSCs in normal culture medium without Retinoic Acid.B:DFSCs in the standard neural inductive medium (Scale bar: 100 um).

The results of studying nerve and bone cells gene expression at mRNA level with RT-PCR technique:

Expression of nerve cell genes (MAP2 and B-tubulin) and bone cell genes (Osteocalcin and Osteopontin) at mRNA level with RT-PCR technique were studied. According to the result expected from primers designed (Table 2), the results of testing by DNA Ladder 100 bp were confirmed. The negative control group of the test containing water showed that there was no contamination of DNA and RNA in response materials.

Medium normal without retinoic acid was not statistically significant in the expression of nerve cells, and in other words, cells of these genes were not expressed in B-tubulin and MAP2. Moreover, normal culture medium cells without BMP2 did not express Osteocalcin and Osteopontin genes. In normal neural culture medium, B-tubulin gene and MAP2 were expressed. Positive control group shows B-tubulin and MAP2 neural differentiation gene expression in NT2 cell (neural progenitor cells). The positive control group shows bone differentiation of Osteocalcin and Osteopontin gene expression in bone precursor cells (Figure6,7).

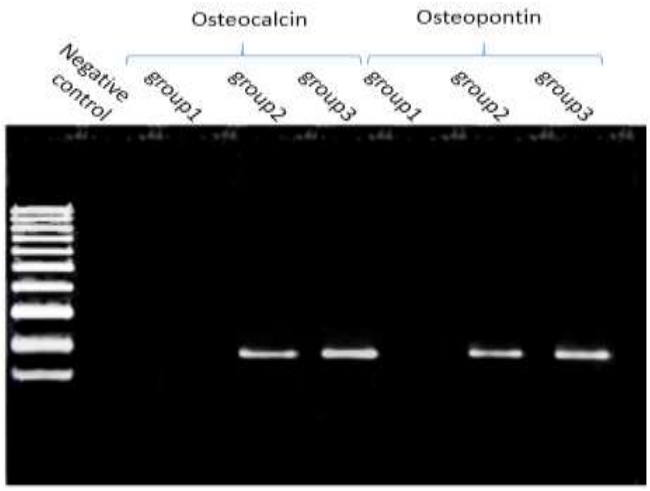

Figure 6: Real-time PCR analysis of DFSCs. Agarose gel Electrophoresis for RT-PCR production of osteocalcin and osteopontin genes in stem cells derived from 3rd molar follicle after 14 days of cultivation in different groups in DNA ladder fermentase 100bp. Negative control: H2O, Group1: normal culture medium without BMP2, Group2: standard osteogenic inductive medium containing BMP2, group3: Positive control.

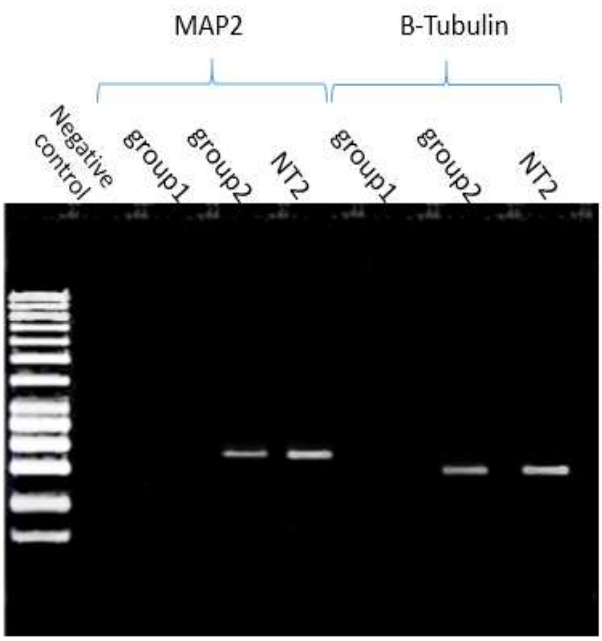

Figure 7: Real-time PCR analysis of DFSCs. Agarose gel Electrophoresis for the RT-PCR production of MAP2 and b-Tubulin genes in stem cells derived from 3rd molar follicle after 21 days of cultivation in different groups in DNA ladder fermentase 100bp. Negative control: H2O, Group1: normal culture medium without retinoic acid, Group2: standard neural inductive medium containing retinoic acid, NT2: Positive control.

The results of examining nerve and bone cells gene expression at protein levels with Western Blott technique:

Expression of protein products of nerve cell genes (MAP2 and B-tubulin) and bone cell genes (Osteocalcin and Osteopontin were analyzed by Western blot technique. The results were consistent with Packer RT- test (Figure 8).

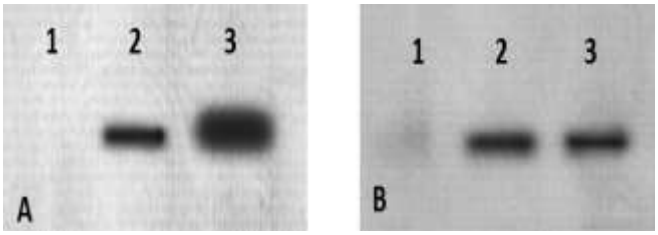

Figure 8: Expression of Osteocalcin protein (A) and Osteopontin protein (B) in DFSCs after 14dayes culture in different mediums.1: Normal culture medium without BMP2, 2: Standard osteogenic inductive medium with BMP2, 3: Positive control.

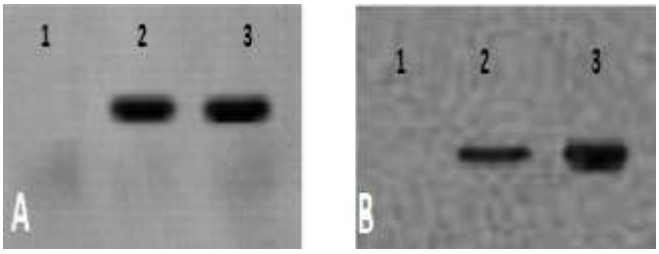

Figure 9: Expression of b-Tubulin protein (A) and MAP2protein (B) in DFSCs after 21 days culture in different mediums.1: Normal culture medium without Retinoic Acid, 2: Standard neural inductive medium with Retinoic Acid, 3: NT2

Measuring alkaline phosphatase enzyme activity

sample 1	Group 1	Group 2	Positive control
DFSCs	0.12	0.59	0.53
	0.114	0.52	0.61
	0.12	0.51	0.62
average	0.118	0.54	0.586666667

Discussion

Today, tissue engineering has found a special place in reform medicine. It seems that the use of autologous stem cells producing different textures common in the future probably will be the best choice for regenerative procedures (22,23).

In this study, stem cells were prepared from the follicles of impacted third molars. Then, using osteogenic and adipogenic differentiation during a 14-day differentiation protocol, stem cell sample was confirmed. In addition, in order to prove the mesenchymal origin of these cells, cell surface markers were analyzed by flow cytometry analysis. Positive expression of CD44, CD90, CD73 and CD105 markers and negative expression of CD34 and CD45 markers are indicative of mesenchymal cells. The results of flow cytometry test obtained were consistent with the study of Yalvac (24) and Mashhadi Abbas (25). These studies determined the nature of stem cells isolated from human third molars and examined CD and confirmed mesenchymal markers of the cells.

According to the results, it was demonstrated that dental follicle tissue has pluripotent stem cells. These results also can show the ability of these cells to differentiate into more specialized cell lines and suggest the possibility of using tissue engineering. The results of the present study were similar to previous studies which stated DFSCs was able to differentiate into specialized cell lines of at least into two layers of different embryonic origins (26). After that, specifically differentiations of bone and nervous DFSCs were examined. Bone mineral matrix is along with human osteoblasts. Active adult osteoblasts cells of the bone are responsible for building charged mineral matrix.

Alizarin Red staining being positive confirmed the formation of calcified nodules in cell matrix (27) and differentiation of mesenchymal cells into osteoblasts (28). The results of this study showed that calcified nodules were formed after differentiation in osteogenic standard culture medium containing BMP2. These calcified areas became red combined with Alizarin Red. As we know, a calcified matrix of bone cells is caused by deposition of calcium and the presence of phosphate is essential for calcium entry into the cell (29, 30). For this purpose, we need alkaline phosphatase enzyme activity. Alkaline phosphatase is one of the main factors in the process of osteogenic differentiation.

Measuring the activity of this enzyme is considered as one of the main factors in differentiating into bone (31). In this study, the activity of this enzyme was measured in the test and control groups. In osteoblast cells different genes are expressed, some of which are osteopontin, osteocalcin, Bmp one and two, alkaline phosphatase, etc. (30). In this study, like a similar study by Eslaminejad et al., (32) the expression of the gene for osteopontin and osteocalcin was used differentiation and tested to confirm the RT-PCR and Western blot of these genes expression were confirmed. In a study, Abnusi et al. examined the mesenchymal stem cells morphology of bone marrow before and after their osteogenic differentiation. In this study, osteogenic differentiation was assessed by staining Hoechst and acridine orange.

In addition, the matrix deposition, calcium, alkaline phosphatase enzyme activity and metabolic activity of differentiated cells by MTT assay were examined (33). Tests of the two studies were different but in the end, all these tests showed the ability of osteogenic differentiation of stem cells. In another study, Mori et

al. examined DFSCs osteogenic differentiation by measuring alkaline phosphatase activity and gene expression RUNX1 and RUNX2 where the positive expression of these genes in differentiated cells confirmed bone formation (34).

Mesenchymal stem cells, according to their origin that is from neural crest, have high potential to differentiate into neural cell lines (35). DFSCs neuronal differentiation was carried out by morphology and gene expression and MAP2 B-Tubulin. The results of the mentioned experiments were positive and confirmed neuronal differentiation. In a study, Imran Ullah et al. compared neural differentiation of dental tissues. Studied genes for neural differentiation in the study by Ullah included MAP2 and B-Tubulin like the present study. In addition, NF-M and NGF genes were also examined. Finally, the ability of neural differentiation of stem cells, pulp, PDL, and human dental follicle was confirmed by the positive expression of these genes (36). In addition, in another study, they compared the differentiation potential of neural stem cells derived from dental pulp and periodontal ligament, and like the present study, by studying the expression of MAP2 and B-Tubulin, they confirmed neuronal differentiation of these cells (37). According to DFSCs potential to differentiate into a variety of cell lines including neural tissue and bone, it seems that these cells are a good source of stem cells that can be used to repair nerve damage and cell therapy-based treatments, especially in the area of maxillofacial bone defects. These cells can easily, and at a low cost, be prepared and can play an important role in the future of cell therapy.

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