RESEARCH ARTICLE

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Neural Differentiation of Wisdom Tooth Follicle Stem Cells on a Nano-Hydrogel Scaffold Containing Salvia Chloroleucat to Treat Nerve injury in the Cancer of Nervous System

Naghmeh Bahrami[1,2], Zeynab Manafi[2], Farnoush Mohammadi[3], Seyedeh Zahra Fotook Kiaei[4], Abdollah Farhadi Nasab[5], Seyed Hossein Hosseini Largani[1], Meraj Naghdi[6], Abdolreza Mohamadnia[7,8]*

Abstract

The human third molar's follicle is one of the sources of stem cells with high differentiation capacities which can be used in nervous system cancer treatment particularly in nerve damge. The purpose of this research was to identify the effects of the aqueous extract of Salvia chloroleuca on the differentiation of the human dental follicle-derived mesenchymal stem cells to neural cells for treti. In this experimental study, the method of culture of digested tissue fragments was used to isolate stem cells from three samples of the extracted wisdom teeth follicles. The nano-hyaluronic acid scaffold has been synthesized by the sol-gel method as a porous composite and the *S. chloroleuca* extract has been loaded into it. The scaffold was analyzed in terms of mechanical properties, drug release and toxicity. Afterwards, the cells were seeded onto the scaffold using the immersion method. After 21 days, cell differentiation was investigated by morphological confirmation methods and confirming the expression of β-tubulin and MAP2 genes at mRNA and protein levels. Morphological assessment revealed neural differentiation in the cells of the groups of nano-hyaluronic acid scaffold with *S. chloroleuca* extract and nano-hyaluronic acid scaffold with *S. chloroleuca* extract + 10% retinoic acid. Furthermore, the expression of MAP2 and β-tubulin in these groups was confirmed by RT-PCR, real time PCR and western blot assays. The results of this research showed that the follicle of the third molar contains stem cells with a high capacity for differentiation. Moreover, the extract of S. chloroleuca, could lead to induction of neural differentiation in stem cells.

Keywords: Stem cell- follicle- wisdom tooth- S. chloroleuca- neural differentiation

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Introduction

Cancers of the central nervous system can cause irreparable complications due to damage of nerve cells and lack of access to the tumor(Pentsova et al., 2016) Moreover, the nerve cells do not have reproductive power after birth, the repair of nerve lesions is one of the most complex treatments (Pisciotta et al., 2020). Nowadays, cell treatments based on tissue engineering and stem cells are greatly appreciated in regenerative medicine (Chun et al., 2018). Nerve tissue engineering is also a suitable option for treating nerve injuries and lesions specially in cancer (Ding et al., 2019; Kline-Quiroz et al., 2020). The human dental follicle (DF) is a tissue which is part of the tooth bud. This tissue is derived from the mesenchyme of the tooth, and like other tissues of the dental bud (enamel organ or dental papilla), it disappears during the development of the tooth. Contrary to the enamel organ, DF can be isolated from the human third molars after extraction. Human DF has been shown to contain mesenchymal stem cells, which can be considered a source of stem cell-based treatments (Bastos et al., 2022; Lei et al., 2021). One of the applications of stem cells capable of differentiation into neural cells is the treatment of cancers

*[1]Department of Tissue Engineering, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, Iran. [2]Craniomaxillofacial Research Center, Tehran University of Medical Sciences, Tehran, Iran. [3]Oral and Maxillofacial Surgery Department, School of Dentistry, Tehran University of Medical Sciences, Tehran, Iran. [4]Department of Pulmonary and Critical Care, Shariati Hospital, Tehran University of Medical Sciences, Tehran, Iran. [5]Behavioral Sciences Research Center, Taleghani Hospital, Shahid Beheshti University of Medical Sciences, Tehran, Iran. [6]Islamic Azad University of Medical Sciences, Tehran, Iran. [7]Chronic Respiratory Diseases Research Center, National Research Institute of Tuberculosis and Lung Diseases (NRITLD), Shahid Beheshti University of Medical Sciences, Tehran, Iran. [8]School of Advanced Technologies in Medicine, Department of Biotechnology, Shahid Bheshti University of Medical Sciences, Tehran, Iran. *For Correspondence: mohamadnia.ar@gmail.com. Seyed Hossein Hosseini Largani and Abdollah Farhadi Nasab have equal contribution in this study.*

specially brain tumors (Bagó et al., 2016). Because of the prevention of the blood-brain barrier, the administration of drugs to brain tumors is one of the challenges in the treatment of this type of cancer. Consequently, new therapeutic approaches like stem cells hold promise in this regard (Ahmed et al., 2010; Young et al., 2014).

In order to enhance the effectiveness of cell and tissue culture in tissue engineering, the three-dimensional conditions of the body (in vivo) should be created in the external state (ex vivo). In order to achieve this, the desired tissue cells are cultured on scaffolds. These structures have the ability to mimic and support the three-dimensional tissue structure. To be clear, the most important part is the design of the scaffold, where hole size, porosity intensity and degree of destructibility are determined (Cheung et al., 2007; Yu et al., 2019). One of the largest and most effective scaffolds used for stem cell culture and tissue engineering is hydrogel scaffold (Lin et al., 2019).

A further pillar of tissue engineering is the growth factors that, by being present in the culture medium, induce the pathway of differentiation in stem cells. Growth factors (GFs) are natural molecules with a structure of protein or steroids that cause cell proliferation and differentiation. They serve to regulate different cellular processes. Certain natural compounds such as herbal extracts may have the same function as GFs. Plants belonging to the genus Salvia have medicinal properties on the central nervous system. Along with its neuroprotective effect on the nervous system, can be attributed to antioxidant, anti-inflammatory, antipyretic, sedative and hypnotic effects, skeletal muscle relaxant, treatment of many indicated diseases including colds, bronchitis, digestive disorders, tuberculosis, and skin and nervous diseases (Cho et al., 2007; Juergens et al., 2003). *S. chloroleuca* has been shown to play an effective role in inhibiting seizures caused by pentylenetetrazole and in preventing the development of epilepsy in mice (Behnam et al., 2000). In addition, salvia extract is used to improve cognitive function in young adults by establishing cholinergic properties (Tehranipour et al., 2010). Results from several studies indicate that *S. chloroleuca* has antioxidant, anti-inflammatory, analgesic, anti-apoptotic and antimicrobial properties (Alamdary et al., 2012). Considering that to date, no research has been conducted in the field of studying the effect of aqueous extract of *S. chloroleuca* leaf on stem cell differentiation, consequently, we decided to carry out a study to determine the effects of the aqueous extract of *S. chloroleuca* as growth factors on the differentiation of stem cells derived from the follicles of the human third molar to nerve cells in vitro.

Materials and Methods

Isolation and primary cultivation of stem cells

The wisdom teeth used in this study were prepared surgically by three patients following written consent. This study was approved by Ethics committees of Tehran University of medical science(TUMS), Tehran, Iran, (IR.TUMS.REC.1394.1438). Patients between the ages of 15 and 30 were included in the study ensuring the absence of infectious and systemic diseases and the use of drugs, cigarettes and alcohol. After removing the wisdom tooth, the follicle tissue in the crown part of the tooth was separated and rapidly transferred to the laboratory in Hank's solution. In the laboratory, the sample was washed repeatedly with PBS (Phosphate Buffer Saline) containing antibiotics. Then the target tissue was placed in the tissue solvent containing DMEM (Dulbecco's Modified Eagle Medium) and collagenase type 1 enzyme with a concentration of 250 u/ml for 1-2 hours in a shaker incubator at 37 °C and 5% CO2 for tissue digestion. To remove undigested tissue fragments and existing impurities, filtration was carried out through 40 and 70 µm filters. Ficoll was then used to remove mononuclear cells from the sample. The solution containing the sample was centrifuged (eppendorf centrifuge 5810R) for 5 minutes at 1500 rpm to form a cell pellet. The cells were then cultured into a T75 flask containing DMEM culture medium and 10% FCS.

Assessment of the cell stemness

In order to prove the stemness of the cells, they were differentiated into adipocyte and osteoblasts using differentiation culture media. To this end, the cells were cultured in the number of 20,000 cells/ml in a 24-well plate containing DMEM/F-12 medium and 10% serum. After 24 hours, the fat and bone differentiation medium were added to the cells. The adipose differentiation medium included DMEM/F-12 containing 50 µg/ml ascorbic acid 3-phosphate, 100 ng dexamethasone and 50 µg/ml indomethacin. In the bone differentiation medium, 10 mM beta-glycerol phosphate was used instead of indomethacin. The cells were stored in these induction media for 14 days and the culture media was changed three times a week. Oil Red and Alizarin Red staining was used to confirm the differentiation into fat and bone tissue, respectively.

Validation of Mesenchymal Stem Cell

Flow cytometry analysis was used to investigate the phenotypic profile of surface markers of stem cells. For this, 105 cells were suspended in a volume of 100 ml of PBS. Antibodies CD44-FITC, CD90-FITC, CD45-FITC, CD73-PE, CD34-PE, CD105-PE (Exbio/Czech) were added in an amount of 5 µl. Mouse isotype control antibodies IgG1-FITC, IgG1-PE, and IgG2a-PE were added to the 105 cells in separate tubes. The tubes were incubated at 4°C for 30 minutes in a dark room. The cells then were mixed with 1 ml of washing buffer and centrifuged at 1500 rpm for 5 minutes. Each cell sample was suspended in 250 µl of washing buffer and immediately read by BD FACSCalibur flow cytometry (BD bioscience USA, CA, San Jose). The obtained data were analyzed in Flowjo.7.6.1 software.

Preparation of S. chloroleuca extract

The leaves of *S. chloroleuca* were obtained from the Cellular and Molecular Research Center of Gandhi Hospital-Tehran, Iran. The aqueous extract was obtained from powdered leaves using a Soxhlet (model H626). The solvent was removed with a rotary evaporation.

Preparation of nano-hyaluronic scaffold

To make the scaffold, the hydrogel was made in a

nano-sized porous mixture by the sol-gel method. The *S. chloroleuca* extract as a differentiating factor was loaded into the porosity of the scaffold at a concentration of 30%. 0.54 ml of hyaluronic acid (HA) solution was dissolved into 15 ml of isooctane containing 0.2 M AOT and 0.04 M 1-HP. Divinyl Sulfone (DVS) as a cross linker was added to the solution. This solution was filtered using Whatman paper and put into acetone. Particles larger than eight microns were filtered. Two phases were formed and the organic phase containing isooctane and acetone was discarded and the precipitate was dissolved in acetone again. The particles were separated by centrifugation and dried at 25°C. The dried gel was powdered and heated for calcination. The *S. chloroleuca* with a concentration of 30% was loaded in nano-hyaluronic acid scaffold by the double emulsion method.

Scaffold characterization

Mechanical test of the scaffold: Before seeding the cells, the scaffolds were examined for mechanical tests. To achieve this, SEM microscopy was used. The mechanical properties include Tensile Young's modulus, Compression Young's modulus and Complex shear modulus were obtained using uniaxial compressive, uniaxial tension and direct shear tests.

Drug release test: To assessment of the drug (*S. chloroleuca* extract) release from the synthesized scaffold, first, nano-hyaluronic acid was freeze-dried, weighed and resuspended into the PBS at a pH 5.6. It was incubated in a water bath at 37°C. The *S. chloroleuca* extract-carrying nano-hydrogel in a dialysis bag was immersed in PBS, and dialysis was performed. The *S. chloroleuca* extract release was read by measuring the absorbance of the released extract using a spectrophotometer for at 570 nm.

Toxicity test: The cytotoxicity of nano-hyaluronic acid scaffold for NT2 (neural progenitor cells) and HEK293T (Human Embryonic Kidney Cells) cell lines was investigated using the MTT assay. The cells were cultured quadruplicate in a 96-well plate (about 3000 cells in each well containing a scaffold). After 72 hours of cell culture on the scaffold, the content of each well at a wavelength of 570 nm compared to a reference wavelength of 630 nm was read using an ELISA reader and the percentage of cell viability was calculated.

Seeding the cells on the scaffold

Cells were seeded on the scaffold by immersion method. After that, the study was conducted in 5 groups: (1) stem cells seeded on nano-hyaluronic acid scaffold without *S. chloroleuca* extract, (2) stem cells seeded on nano-hyaluronic acid scaffold with *S. chloroleuca* extract, (3) stem cells seeded on scaffold nano-hyaluronic acid with *S. chloroleuca* extract + 10% retinoic acid, (4) stem cells was cultured in DMEM + FBS 10%, and (5) stem cells was cultured in the standard neural culture medium. The standard neural culture contained dexamethasone 10ng, ascorbic acid 50 µg/mL, retinoic acid phosphate 10mMol.

Assessment of neural differentiation

After 14 days, cell differentiation was studied by methods for confirming morphology and expressing specific genes in neural cells. Morphological confirmation: The cells in the two-dimensional differentiation culture medium were examined by optical microscope and the cells in the three-dimensional hyaluronic acid scaffold were examined in terms of morphology by SEM.

β-tubulin and MAP2 expression at the mRNA level: Analysis of β-tubulin and MAP2 expression in mRNA level was performed using qRT-PCR and real-time PCR. RNA extraction of the cultured cells on the scaffold containing *S. chloroleuca* extract was performed using the RNXTM (PLUS) kit and according to the manufacturer's instructions. The quantitative analysis of extracted RNA was evaluated using an ultraviolet spectrophotometer at wavelengths of 230, 260 and 280 nm. AccuPower® RocketScript™ RT PreMix kit from Bioneer was used for cDNA synthesis. Then, 1 µg of synthesized cDNA, 2 µl of PCR buffer 10X, 1 µl of 0.5 mM dNTP, 0.75 µl MgCl2 and 2 µl of each gene-specific primer (Table 1) were mixed together at a final volume of 20 µl to run the qRT-PCR and real-time PCR. The PCR product was loaded on a 1% agarose gel. The GAPDH reference gene was used for data normalization.

β-tubulin and MAP2 expression at the protein level: The expression of MAP2 and B-tubulin proteins was evaluated using western blot analysis. After 14 days of culture on the scaffold, the cells were digested with 12.5% trypsin at 37°C for 35 seconds and centrifuged at 4°C and 12000 g for 5 minutes. The cells were dissolved in RIPA buffer (50 mM pH 7.5 Tris-HCl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) and then washed. They were ultrasonicated at 50 Hz and 4 °C. Total protein concentration was detected according to the manufacturer's protocols of the BCA assay kit, and 50 µg of each sample was subjected to 10% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked using 5% skim milk for 1 hour at room temperature. It incubated with primary antibodies (Table 2) overnight at 4°C. Following the incubation with HRP-labeled secondary antibody, they were washed at 37 °C. The protein bands were visualized using Advanced Chemiluminescence Western Blot Substrate Kit (Biovision, USA). The Quantity-One v4.6.6 software was used to analyze the corrected relative protein expression with the internal β-actin reference (1:1000 dilution, Abcam ab8227).

Results

Assessment of the cell stemness

To prove the differentiation capacity in vitro, wisdom tooth follicle stem cells were placed in the adipogenesis and osteogenesis media. After 14 days, all cells successfully differentiated into adipocytes and osteoblasts. The formation of intracellular lipid droplets in adipocytes was proven by oil red staining (Figure 1-A). The criterion for osteogenesis was the formation of mineralized nodules and calcium deposits, which was proved by alizarin red staining (Figure 1-B).

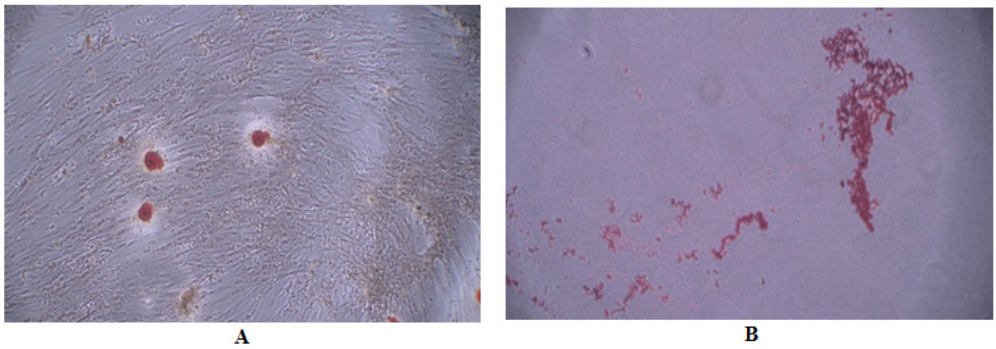


Figure 1. Differentiation Potential to Adipocyte with Oil Red Staining (A) and osteoblasts with Alizarin Red staining (B)

Assessment of mesenchymal cells

Flow cytometry results showed these cells to be positive for mesenchymal markers CD44, CD73, CD90 and CD105 and negative for haematopoietic markers CD34 and CD45. These markers had an expression percentage of 99.2% for CD44, 97.6% for CD105, 99.8% for CD90, 99.6% for CD73, 0.845% for CD34 and 0.587% for CD45.

Scaffold characterization

Evaluation of the electron microscope images revealed that the nano-hyaluronic acid scaffold has an appropriate porosity for drug loading and cell culture (Figure 3-A). The results of the cytotoxicity test after 72 hours on NT2 and HEK293T cells showed that at concentrations of 0.01, 0.1, 5 and 10 µg/µl, the viability of the cells did not decrease significantly compared to the control sample (Figure 3-B). According to the obtained results and the necessary conditions for the construction of the scaffold,

Table 1. Sequence of Primers Used in qRT-PCR

Gene	Primer Sequence	Product Length (bp)
β-Tubulin	F: GGAGGGGCATCTCTTGAGAAC	273
	R: TCGAGGCACGTACTTGTGAG	
MAP2	F: GCTCCCGGAGAAGGATTCTG	329
	R: TCAGGTGGATGTGAGTGTGC	

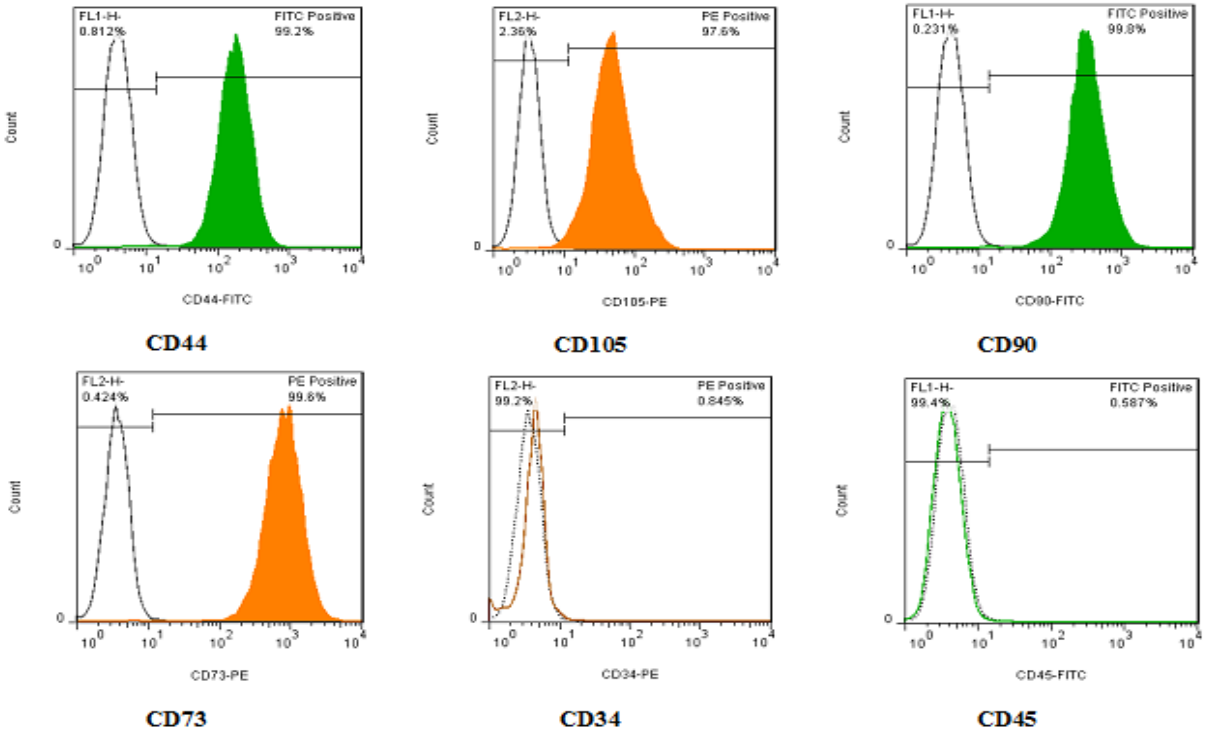


Figure 2. Flow Cytometry Histogram of the Expression of Mesenchymal and Hematopoietic Markers with the Control Isotype Sample

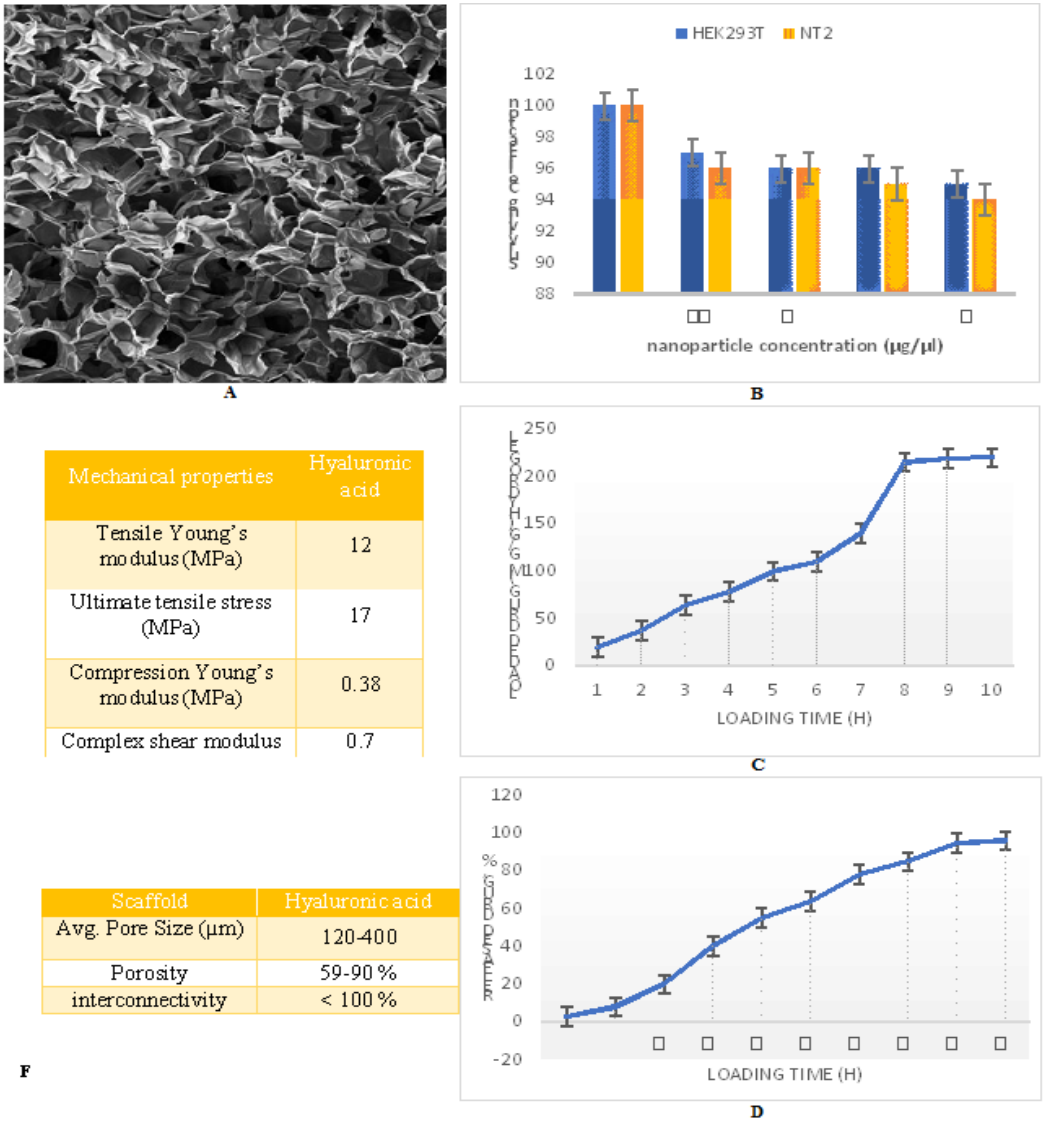
Table 2. Primary Antibodies Used for Western Blot

Antibody	Description	Molecular weight (KDa)
Anti-MAP2	Chicken polyclonal to MAP2(ab5392)	280
Anti-beta tubulin	Goat polyclonal to beta Tubulin(ab21057)	51
Anti-beta actin	Rabbit polyclonal to beta Actin(ab8227)	-

a concentration of 5 µg/µl of nano-hyaluronic acid was selected for use in the scaffold. Drug release test results showed a gradual release of the drug within 45 hours, up to 96% of the loaded extract (Figure 3-C). It was found that firstly, the release of the extract was gradual in 5-hour time intervals, which has a critical role for the use of scaffolds in cell differentiation. Secondly, the release of 96% of the loaded drug shows that the synthesis of the nano-hyaluronic acid scaffold is correct and confirms its biodegradability and the possibility of drug release (Figure 3-D). The mechanical properties include Tensile Young's modulus, Compression Young's modulus and Complex shear modulus were performed for the scaffold (Figure 3-E). Also, the results of examining the porosity and characteristics of scaffold pores are shown in Figure 3-F, which all show that the scaffold is suitable for use in cell differentiation and drug loading with the appropriate mechanical characteristics.

Morphological evaluation of the cells on the scaffold

The cell morphology after differentiation were analyzed with an optical microscope in the group of standard neural culture media containing retinoic acid (Figure 4-A) and the group of normal culture media



Mechanical properties	Hyaluronic acid
Tensile Young's modulus (MPa)	12
Ultimate tensile stress (MPa)	17
Compression Young's modulus (MPa)	0.38
Complex shear modulus	0.7

Scaffold	Hyaluronic acid
Avg. Pore Size (µm)	120-400
Porosity	59-90 %
interconnectivity	< 100 %

F

Figure 3. Evaluation of the Synthesized Scaffold Containing *S. chloroleuca* Extract (A: SEM microscope images of nano-hyaluronic acid scaffold, B: MTT diagram in 72 hours for nano-hyaluronic acid scaffold, C: drug loading in nano-hyaluronic acid scaffold, D: drug release of nano-hyaluronic acid scaffold, E: physical properties of nano-hyaluronic acid scaffold and F: results of nano-hyaluronic acid scaffold mechanical tests)

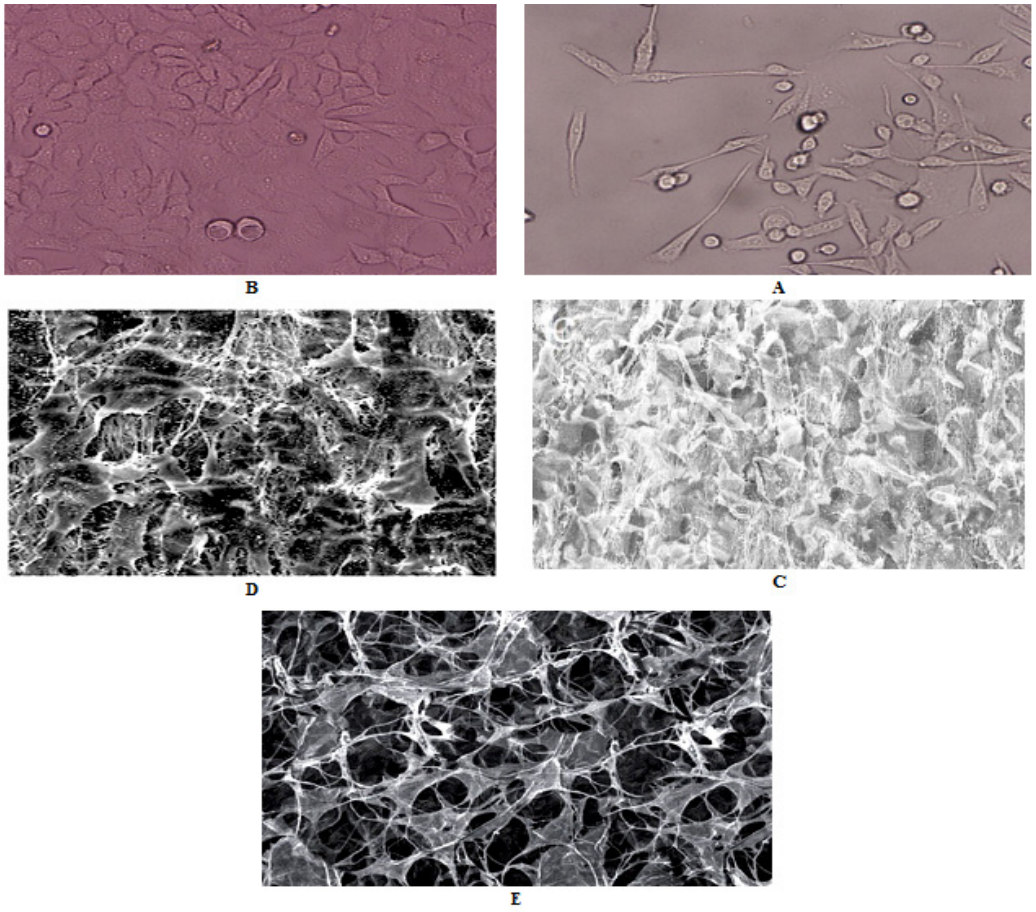


Figure 4. Morphological Evaluation of Cultured Cells in Five Studied Groups, Including Stem Cells in Standard Neural Culture Medium (A), negative control cells cultured in DMEM+FBS 10% medium (B), stem cells seeded on nano-hyaluronic acid scaffold without *S. chloroleuca* extract (C), stem cells seeded on nano-hyaluronic acid scaffold with *S. chloroleuca* extract (D), stem cells seeded on nano-hyaluronic acid scaffold with *S. chloroleuca* extract +10% retinoic acid (E)

(Figure 4-B). While in the groups of nano-hyaluronic acid without *S. chloroleuca* extract (Figure 4-C), nano-hyaluronic acid with *S. chloroleuca* extract (Figure 4-D) and nano-hyaluronic acid group with *S. chloroleuca* extract + 10% retinoic acid (Figure 4-E) were evaluated with SEM microscope. As can be seen in the Figure 4, no differentiation occurred in the cells seeded on the nano-hyaluronic acid without *S. chloroleuca* and on the normal culture media without retinoic acid, and the morphology of neural cells did not appear. In the nano-hyaluronic acid group containing *S. chloroleuca* extract and the nano-hyaluronic acid group with *S. chloroleuca* extract + 10% retinoic acid, the cells completely show the morphology of neural cells. In the standard neural culture medium, the cells are differentiated into neural cells.

MAP2 and β-tubulin expression

The expression of MAP2 and β-tubulin genes was investigated at the mRNA level by RT-PCR and Real time PCR techniques and at the protein level by western blot method. The results of RT-PCR were shown in Figure 5-A. It can be seen that β-tubulin and MAP2 genes were not expressed in the cells on nano-hyaluronic acid scaffold without *S. chloroleuca* extract (column 1) and on the normal culture medium without the differentiation factors (column 4). In the cells on the nano-hyaluronic acid scaffold with *S. chloroleuca* (column 2), the nano-hyaluronic acid scaffold with *S. chloroleuca* extract + 10% retinoic acid (column 3) and the standard neural culture medium (column 5), β-tubulin and MAP2 genes were expressed. In the cells on standard neural culture medium (column 5), the most gene expression was observed. Column 6 is also the positive control group which shows the expression of β-tubulin and MAP2 in NT2 cells (neural progenitor cells). Real time PCR analysis was also completely consistent with these findings (Figure 5-B and 5-C) and showed that the expression of β-tubulin and MAP2 in the cells on nano-hyaluronic acid scaffold without *S. chloroleuca* extract (column 1) and on the normal culture medium without the differentiating factors (column 4) is close to zero. While in the cells on the nano-hyaluronic acid scaffold with *S. chloroleuca* extract (column 2), the nano-hyaluronic acid scaffold with *S. chloroleuca* extract + 10% retinoic acid (column 3) and the standard neural culture medium with differentiation factors (column 5), β-tubulin and MAP2 were expressed. The expression of both genes was also observed in the positive control group (column 6). The expression of MAP2 and β-tubulin at the level of protein was investigated by western blot technique

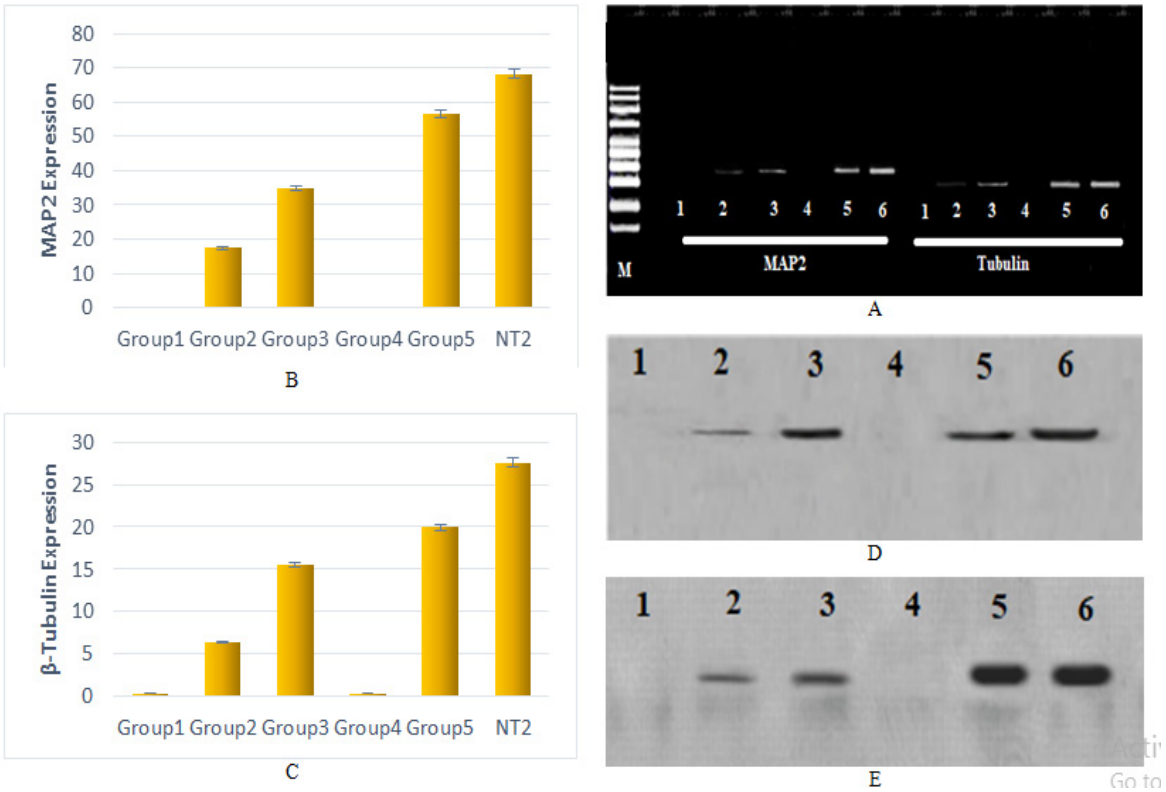


Figure 5. Evaluation the Expression of MAP2 and β-Tubulin Genes in the Human Dental Follicle-Derived Mesenchymal Stem Cells after 14 Days of Culture in Different Groups by qRT-PCR Method (A), Real time PCR (B: MAP2 and C: β- Tubulin) and western blot (D: MAP2 and E: β-Tubulin). In all figures, column 1: the nano-hyaluronic acid scaffold without *S. chloroleuca* extract, column 2: the nano-hyaluronic acid scaffold containing *S. chloroleuca* extract, column3: the nano-hyaluronic acid scaffold containing *S. chloroleuca*extract + 10% retinoic acid, column 4: normal culture medium without the presence of differentiation factors, column 5: standard neural culture medium with differentiation factors, column 6 (NT2): positive control group containing NT2 cells. M: 100 bp DNA ladder

(Figures 5-D and 5-E, respectively). The results obtained were similar to the results of RT-PCR and Real time PCR. In general, the highest expression was observed in the positive control group (column 6). The closest level of expression to this group was in the cells on the standard neural culture medium with differentiating factors (column 5). After that, the cells on the nano-hyaluronic acid scaffold with *S. chloroleuca* extract + 10% retinoic acid (column 3) and the nano-hyaluronic acid scaffold with *S. chloroleuca* extract (column 2) respectively had the highest MAP2 and β-tubulin expression.

Discussion

In recent years, mesenchymal stem cells (MSCs) have been seen as a cancer management strategy. Besides the role in the regeneration of damaged nerve tissue, modulation of the immune system, anti-inflammatory effects, and bioactive molecules secretion, MSCs have great potential as antitumor agents (Belmar-Lopez et al., 2013; Gjorgieva Ackova et al., 2016; Pessina et al., 2011; Santiago-Osorio et al., 2016). Systemic injection of MSCs has been shown to reduce tumor growth, indicating the effect of inhibition of tumor proliferation (Gjorgieva et al., 2013; Petrella et al., 2017). MSCs not only inhibit tumor growth, but can also act as carriers of antitumor agents (Bonomi et al., 2017; Bosco et al., 2015; Kéramidas et al., 2013).

Dental pulp is an adequate source of mesenchymal stem cells that have recently been the focus of attention because of the abundance of cells in each tooth and convenient isolation methods from other tissue sources of stem cells (Collart-Dutilleul et al., 2015). Pulp tissue of human third molar teeth is an available source of MSCs. The properties of dental pulp stem cells distinguish them as one of the most accessible cell sources for cell-based therapy (Santiago-Osorio et al., 2016).

Therefore, human third molar's follicle derived MSCs were utilized in this study. The stemness and mesenchymal features of the isolated cells were confirmed through specific staining and investigation of specific markers, respectively. In similar studies, the isolation of MSCs from the dental pulp was also discussed. Nikkhah et al., (2021) extracted the dental pulp stem cells and investigated their inhibitory effect on the development of colorectal cancer. Their results showed that the mentioned cells induce apoptosis and reduce the viability of colorectal cancer cells. Salehi et al., (2018) showed that dental pulp stem cells are capable to deliver the anticancer drug paclitaxel to the target cells.

The use of dental stem cells for targeted cancer treatment may be a revolution in reducing chemotherapy-induced complications and increasing the effectiveness of systemic cancer treatment. On the other hand, providing the conditions for their differentiation is also an important issue that depends on various factors such as the scaffold

used and compounds stimulating differentiation. In this research, nano-hyaluronic acid scaffold and *S. chloroleuca* extract were used to induce neural differentiation of stem cells derived from third molar teeth. The results showed that the differentiation was correct and that the scaffold and inductive agent used had an appropriate performance.

So far, a variety of studies have been conducted in the field of cell scaffold adapted to stem cell culture and differentiation into different cell lines. The spread of stem cells in three-dimensional scaffolds facilitated the cell development process and provided high-quality multipotent cells for broad-spectrum use in regenerative medicine (Murphy et al., 2020). Furthermore, hyaluronic acid hydrogel scaffold was introduced as an appropriate environment for neural differentiation of stem cells. One such scaffold is the hyaluronic acid hydrogel, which is used for stem cell culture and neural differentiation due to its proper structural characteristics. In the present study, hyaluronic acid nano-hydrogel scaffold was used for cell culture (Agarwal et al., 2020; Ahmadian et al., 2019). In the study of Bian et al., intelligent self-crosslinking hyaluronic acid hydrogels were introduced as three-dimensional injectable scaffolds for cell culture. In this study, new intelligent self-crosslinking controllable hydrogels with in situ gelation feature were made as a single component by thiolated hyaluronic acid (HA-SH) and as a three-dimensional scaffold to simulate the natural extracellular matrix for fibroblast (L929) and chondrocytes cell culture. The results showed that HA-SH hydrogels with controllable coagulation process, intelligent degradation behavior, excellent biocompatibility and suitable features for convenient use, clinical use capacity for engineering have provided tissue and regenerative medicine (Bian et al., 2016). In the present study, instead of using HA-SH, a nano-hydrogel scaffold with hyaluronic acid was used, in which the extract of *S. chloroleuca* was loaded. The scaffold loaded with the extract has been successfully used as a culture medium for neural differentiation of stem cells from the follicle of the wisdom tooth.

In the study of Xu et al., (2015) enzyme- mediated hyaluronic acid-tyramine (HA-Tyr) hydrogels were investigated for the propagation of human embryonic stem cells (hESC) in 3D. HA-Tyr hydrogels were formed by cross-linking of equal parts of tyramine with Horseradish Peroxidase (HRP) and hydrogen peroxidase (H_2O_2). By changing the concentration of HRP and H_2O_2, HA-Tyr hydrogels with different mechanical resistance were created and the self-renewal characteristics of hESCs in these scaffolds were studied. It was observed that the mechanical strength and chemical composition of the substrates were two important factors that affected cell proliferation. Immunohistochemical analyses indicated that hESCs proliferated well and formed 3D spheroid structures without apoptosis. The hESCs cultured in HA-Tyr hydrogels showed over expression of CD44 and pluripotency markers. These cells have demonstrated the ability to form cells from the three major embryo layers in vitro and in vivo. In addition, the genetic integrity of hESCs remained unchanged in the 3D culture system (Xu et al., 2015). In the current study, only HA was used in the nano-hydrogel scaffold and no other substances were added. Similarly, as in the Xu et al., (2015) study, it was observed that the scaffold provided appropriate and ideal conditions for the growth, proliferation and differentiation of stem cells.

S. chloroleuca is a type of plant of the mint family, the extract of which has many properties. One of the most important effects of *S. chloroleuca* extract is that it affects the nervous system. Salvia plants have medicinal properties and neuroprotective effects on the central nervous system. In the study of Razavi et al., (2014) they reported the neuroprotective effects of the aqueous extract of *S. chloroleuca* on the degeneration of alpha motor neurons of the spinal cord after compression of the sciatic nerve in rats. Therefore, these effects are probably caused by the presence of growth and repair factors in the aqueous extract of the *S. chloroleuca* leaf, which promotes the regeneration process in damaged neurons and prevents the severity of degeneration (Razavi et al., 2014). In this study, the effect of different doses of the extract that was directly injected into the spinal cord lesion was investigated, while in the present study, *S. chloroleuca* extract report as an inducing factor for neural differentiation of human dental follicle-derived mesenchymal stem cells.

In the study of Zeng et al., (2020) muscle derived stem cells (MDSCs) differentiated into neuron-like cells in vitro using ciliary neurotrophic factor (CNTF) and S. miltiorrhiza extract. They used the MDSCs to repair rat sciatic nerve damage in vivo to investigate their multi-functional properties as pluripotent stem cells. The research showed that the MDSCs differentiated by CNTF and S. miltiorrhiza extract played an active role in the repair of peripheral nerve damage. In this study, CNTF and S. miltiorrhiza extract were used for the differentiation of stem cells in vitro. In this study, stem cells were derived from muscle, while in our study, these cells were isolated from dental follicle tissue. In addition, the extract used in our trial was *S. chloroleuca*, which differs from the study of Zeng et al., (2020). The most important difference is the use of CNTF along with the extract for neural differentiation of MDSCs, which seems to be the main cause of the differentiation of cells into neuron. CNTF is a stimulating cytokine for the production of myelin in the nervous system. It also causes the survival and differentiation of oligodendrocyte progenitor cells in the spinal cord (Zeng et al., 2020). Zeng et al., (2020) mentioned that finally the cells were differentiated into neuron-like cells in vitro, while in our study, after differentiation, the cells showed a clear morphology of neural cells and also the expression of specific genes of neural cells.

Tehranipour et al., (2010) investigated the neuroprotective effect of the aqueous extract of *S. staminea* on the nerve density of alpha motor neurons in the anterior horn of the spinal cord after compression of the sciatic nerve in rats. The results showed a significant increase in the number of neurons. Alpha motor neurons was higher in the groups treated with extract compared to the control group. This indicated that this extract can increase the nerve density of motor neurons in the anterior horn of the spinal cord after sciatic nerve injury. In the present research, it was similarly indicated that the

S. chloroleuca extract may cause the neural differentiation of stem cells derived from the molar tooth follicle.

In conclusion, in the present research, it was shown that human third molar's follicle is a source of stem cells that have the ability to differentiate into neural cells with suitable stimuli and growth conditions. We used the *S. chloroleuca* extract as a differentiation stimulant and nano-hyaluronic acid scaffold to create 3D growth conditions. The results indicated that the synthesized scaffold can provide the conditions for the growth and differentiation of stem cells. It was also found that the *S. chloroleuca* extract has an appropriate ability to induce neural differentiation. The findings of this study can be used in regenerative medicine and the treatment of neurodegeneration problems as well as cancer therapy, which of course requires more research in this field.

Author Contribution Statement

N.B.Z.M and F.M conceived the study. Z.K collected the data. A.M, H.H analyzed the data A.F,A.M and H.H wrote the manuscript and all authors approved this final version of the manuscript for publication.

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Ethical Clearance

This study was conducted at Tehran University of Medical Sciences, Tehran, Iran after receiving the ethical code (IR.TUMS.REC.1394.1438.). It is part of an approved student thesis.

Conflicts of Interest

The authors declare that we have no conflicts of interest.

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