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Original Article

Neurogenic induction of human dental pulp derived stem cells by hanging drop technique, basic fibroblast growth factor, and SHH factors

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ABSTRACT

Background: The progressive destruction of nerve cells in nervous system will induce neurodegenerative diseases. Recently, cell-based therapies have attracted the attention of researchers in the treatment of these abnormal conditions. Thus, the aim of this study was to provide a simple and efficient way to differentiate human dental pulp stem cells into neural cell-like to achieve a homogeneous population of these cells for transplantation in neurodegenerative diseases.

Materials and Methods: In this basic research, human dental pulp stem cells were isolated and characterized by immunocytochemistry and flow cytometry techniques. In the following, the cells were cultured using hanging drop as three-dimensional (3D) and tissue culture plate as 2D techniques. Subsequently, cultured cells were differentiated into neuron cell-like in the presence of FGF and Sonic hedgehog (SHH) factors. Finally, the percentage of cells expressing Neu N and β tubulin III markers was determined using immunocytochemistry technique. Finally, all data were analyzed using the SPSS software.

Results: Flow cytometry and immunocytochemistry results indicated that human dental pulp-derived stem cells were CD90, CD106-positive, but were negative for CD34, CD45 markers ($P \leq 0.001$). In addition, the mean percentage of β tubulin positive cells in different groups did not differ significantly from each other ($P \geq 0.05$). Nevertheless, the mean percentage of Neu N-positive cells was significantly higher in differentiated cells with embryoid bodies' source, especially in the presence of SHH than other groups ($P \leq 0.05$).

Conclusion: It is concluded that due to the wide range of SHH functions and the facilitation of intercellular connections in the hanging droop method, it is recommended that the use of hanging drop method and SHH factor can be effective in increasing the efficiency of cell differentiation.

Key Words: Basic fibroblast growth factor, mesenchymal stem cells, neurogenesis, SHH protein

INTRODUCTION

The development of neurodegenerative diseases such as amyotrophic external sclerosis, Alzheimer's disease, Parkinson's disease, Huntington's disease, and MS can leads to impaired central nervous system

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function. The most important common feature of these abnormal conditions is the removal of a large number of neural and glial cells. In addition, it has been reported that many people all over the world suffer from damage to the nervous system, such as spinal cord injury. Due to the limited ability of central nerve system repair, spinal cord injury can affect the normal life of the individual and can cause many disorders in these people.[1] Therefore, the major studies which performed in the field of neurological damage are based on the nerve regeneration by use of drugs, tissue engineering, and cell transplantation.[2-4] Since the use of drugs such as anti-inflammation and immunosuppressive drugs at an inappropriate time can have adverse effects in the improvement of neurodegenerative disorder, other strategies such as transplantation of stem cells have been welcomed by researchers.[5]

Stem cells are a population of biological cells which are capable to differentiate into other cell types.[6,7] Among them, adult stem cells are a group of multi-potent stem cell that can be isolate from a variety of tissues such as bone marrow, hair follicles, skin, adipose tissues, and dental pulp. Dental pulp plays a major role in tooth homeostasis and is essential for long-term teeth's survival. Dental pulp stem cells (DPSCs) were first isolated in 2002 from human third molar teeth pulp.[8] These cells usually isolated from tissues that are recognized as wastes in dental treatments or jaw and facial surgery. DPSCs are considered as a suitable cell source for cell-based therapies in neuronal lesions because of the lack of ethical problems compared with other stem cell sources such as embryonic stem cells.[9] In addition, these cells can be easily obtained from the tooth pulp and have a high potential for differentiation into nerve cells.[10-12] It is important to note that after stem cell therapy, possible complications such as tumors may be occur due to the progressive proliferation of stem cells, and so the emergence of these possible complications should not be denied.[13] Since the differentiated cells do not have the same proliferation capacity as the stem cells, so the transplantation of differentiated cell instead of stem cells is necessary to reduce possible complications, such as tumorigenicity. In a previous study, DPSCs were differentiated into neuronal cells using resveratrol compounds. The results of this study revealed that this polyphenol is able to advance the neuronal differentiation of stem cells.[10] Similarly, in another research, DPSCs were differentiated into dopaminergic cells with a multistage process in the presence of multiple growth factors (such as BDNF, ascorbic acid, and GDNF). Collectively, the results showed that only about 15% of stem cells were differentiated into dopaminergic cells.[11]

By virtue of this fact that DPSCs can be harvested by minimally invasive procedures and according to the ability of these cells for differentiated into other cell types as well as ability to secrete several neurotrophic factors such as neurotrophin factor 3, neural growth factor, glial growth factor and brain-derived neurotrophin factors, this study was done with aims to provide a simple, effective and short term method for differentiation of human DPSCs (hDPSCs) into neuron cell-like to achieve a homogenous population of neurons.[12]

MATERIALS AND METHODS

Sources of chemicals

Cell culture media and medium supplements were procured from Sigma-Aldrich (USA) or Gibco by Life Technologies (USA). Antibiotics were obtained from Invitrogen by Life Technologies (USA). Ethanol was purchased from Merck Millipore (Germany). All other chemicals were bought from SigmaAldrich (USA) unless stated otherwise.

Isolation of dental pulp stem cells

All procedures used in this basic research, were approved by the Ethics Committee of Islamic Azad University, Khorasgan Branch, Isfahan, Iran (ethics code: IR.IAU.KHUISF.REC.1397.268). After obtaining informed consent, five third molar (wisdom) teeth removed surgically from 20 to 25 years old patients (due to lack of jaw space and ineffectiveness in occlusion) referred to Kellishad Dental Surgery Clinic. Dental pulp detached from the teeth and DPSCs were isolated based on previously described protocol.[14] Briefly, after washing with PBS (to remove cellular debris and blood cells), using a sharp blade, the pulps were gently separated from the crown and digested in a solution of 0.075% collagenase Type I (Invitrogen, UK) for 30 min and then collagenase activity was neutralized using a serum-containing culture medium. After centrifugation at 12,009 g for 10 min; the cellular pellet was dissolved using Medium, and cell suspension was cultured in T25 flask at standard condition.

Cell culture

Two-dimensional and hanging drop

Cells dissociated from a single colony were isolated and sub-cultured as either two-dimensional (2D) or 3D by the hanging drop technique. 2D sub-cultured cells gradually became flattened and acquired a fibroblast-like morphology.

Hanging drop cultures were prepared by placing 20 µl micro-drops of neurobasal media and 10% fetal calf serum containing 20 ng/ml human epidermal growth factor, 40 ng/ml human basic fibroblast growth factor (hbFGF) and 500–700 hDPSCs cells on the lids of 60 mm dishes. Bottom plates of the dishes were filled with PBS to avoid desiccation of samples. Lids were then placed on the bottom plates to achieve hanging drop cultures. Plates are kept in standard conditions for 48 h then embryoid bodies were collected.

Evaluation of human dental pulp stem cells surface markers

For this purpose, 1×10^5 hDPSC cells were used to check CD markers. According to previous study,[15] after washing with BSA/PBS solution, these cells was incubated with respective fluorochrome-conjogated antibodies against CD106, CD90, CD34, and CD45 (Chemicon, Temecula, CA, USA) for 30 min on ice. After washing, cell pellet resuspended in solution buffer and were examined with a fluorescence microscope (BX51, Japan). 200 cells were counted in several fields, and the average percentage of cells expressing each marker was reported. Moreover, flow cytometry analysis was done for cell characterization. For this purpose, after cell fixation with paraformaldehyde (2%), the samples were incubated with respective fluorochrome-conjugated antibodies against CD106, CD90, CD34, and CD45 (Chemicon, Temecula, CA, USA) for 30 min. After cell washing, the cells re-suspended in PBS and finally analyzed using a flow cytometer (Becton Dickinson, San Jose, CA, USA).

It should be noted that this technique was repeated three times, and the results were reported as average.

Differentiation of human dental pulp stem cells into neuron cell-like

Cell differentiation was performed in five groups including; 3D/SHH group which single cells from embryoid bodies were differentiated in presence of 100 ng/ml hbFGF for the first 5 days and 100 ng/ml SHH for the second 5 days, 3D/hbFGF group, which single cells from embryoid bodies were differentiated in the presence of 100 ng/ml hbFGF for 10 days, 2D/SHH

group which the cells obtained from 2D culture, were differentiated in presence of 100 ng/ml hbFGF for the first 5 days and 100 ng/ml SHH for the second 5 days, 2D/hbFGF group, which the cells obtained from 2D culture, were differentiated in presence of 100 ng/ml hbFGF for 10 days, and finally in control group, hDPSCs were cultured in same medium in absence of SHH and hbFGF growth factors for 10 days.

Immunocytochemistry technique for neuronal cell markers

According to previous study,[16] after cell fixation (with 4% paraformaldehyde solution for 20 min), the cells were permeabilized with Triton X100 solution (0.1%) for 1 h and then placed in a blocking solution (BSA = 0.1%/PBS) for 30 min. The incubation was carried out with primary antibodies, including anti-β tubulin III and anti-NeuN, for 60 min at the room temperature. After washing, the samples were incubated with a secondary antibody attached to the FITC for 45 min. Finally, the nuclei were stained with DAPI for 1 min at room temperature and the number of β tubulin III and NeuN positive cells was counted in a minimum total of 200 cells per slide.

Statistical analysis

The comparison of the results was performed using the one-way ANOVA, and the results were reported as mean ± standard error meanwhile, $P \leq 0.05$ was considered to be statistically significant.

RESULTS

Human dental pulp stem cells and embryoid bodies (EBs) characterization

Twenty-four hours after hDPSCs isolation, these cells adhered to the floor of the flakes and began to proliferation and after 2 weeks, the cell confluency was 80%–90%. During this stage, hDPSCs exhibited fibroblast-like morphology. 48 h after hanging drop performing, the cells were aggregated and composed EBs. In addition, immunocytochemistry analysis of hDPSCs revealed that 67% ± 1.3% of them were CD90 positive and 99% ± 1.7% of them were CD106 positive which was significantly higher compared to CD34 (3.5% ± 1.4%) and CD45 (1.5% ± 1.1%) positive cells ($P \leq 0.001$). Moreover, flow cytometry analysis confirmed the results of immunocytochemistry technique [Figure 1].

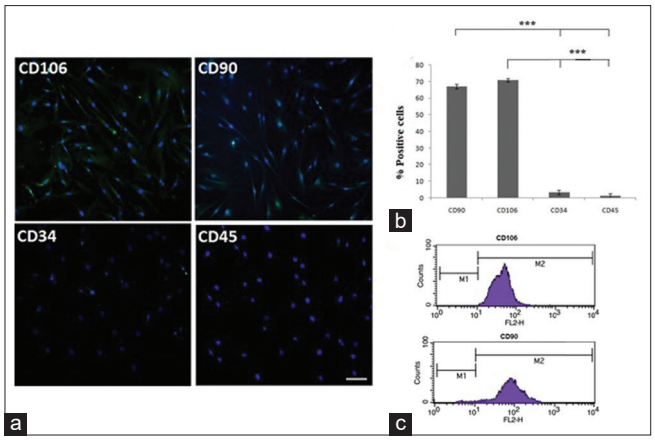

Figure 1: The comparison of CD markers in human dental pulp stem cells. Immunocytochemistry analysis (a) of hDPSCs revealed that these cells were express CD90 and CD106 markers in high level in comparison of CD34 and CD45 (***$P \leq 0.001$) (b). In addition, flow cytometry analysis confirmed the results of immunocytochemistry technique (c). Scale bars represent 200 μm in A. hDPSCs: Human dental pulp stem cells.

Immunocytochemistry results

The comparison of the immunohistochemistry results showed that the mean percentage of - β tubulin III positive cells which differentiated in 3D/SHH and in 2D/SHH groups were 99 ± 0.17 and 96 ± 0.21, respectively, and in 3D/bFGF and in 2D/bFGF groups were 98 ± 0.19 and 9 7 ± 0.29, respectively [Figures 2 and 3], which was not significantly different in comparison with control group (96 ± 0.22) ($P \geq 0.05$). Furthermore, the mean percentage of NenN positive cells which differentiated in 3D/SHH and in 3D/bFGF groups were 17 ± 1.9 and 13 ± 1.3 respectively. In addition, the mean percentage of NenN-positive cells in 2D/SHH and in 2D/bFGF groups were 9 ± 1.4 and 8 ± 1.1, which had a significant decrease compared to the previous group, especially in the presence of SHH compared to bFGF ($P \leq 0.05$) [Figures 3 and 4].

DISCUSSION

In this study, hDPSCs were used to provide a simple, effective, and short-term strategy for differentiation into neuron cell-like in order to achieve a homogeneous population of these cells. After hDPSCs isolation, the mesenchymal features of these cells were proven using immunocytochemistry method. For example, a high percentage of isolated cells showed the specific markers of mesenchymal stem cells, including CD90 and CD106 and showed a lower percentage of hematopoietic stem cell markers. The CD90 which is a glycophosphatidylinositol was first identified in 1980 in thymocytes and then at the level of stem cells, and therefore considered as a marker for the identification of these cells.[17] In addition, CD106 is also a type of sialoglycoprotein that was discovered in 2002 and similar to CD90 was express on the surface of mesenchymal stem cells.[18] In the present study, surface markers and cell morphology results were consistent with the results of Soleimani *et al*. study.[14] With the onset of cell culture, significant morphological changes were observed in these cells. The separated hDPSCs were initially rounded, but over time, their morphology changed and looked like spindle shape similar to fibroblast cells. These morphological changes were intensified by adding growth factors at the stage of cell differentiation.

During the first stage of differentiation process in both methods, various thin cellular processes appeared in different parts of the cell body. Subsequently, these processes became thicker and at the end of differentiation period, a network of differentiated cells with woven processes was observed. In order to evaluation of neural differentiation, we used neuronal cell markers including β tubulin III and NeuN.

β tubulin III was first identified in 1999 by Williams *et al*. This protein is a member of microtubular proteins family and is considered as a marker of immature nerve cells.[19] The expression of this marker alone does not indicate that the cell is necessarily nervous, and therefore, other neuronal markers should be considered in this regard. As shown in Figures 2 and 3, the mean percentage of differentiated cells which expressed β tubulin III marker is not significantly different compared to hDPSCs. In other words, the expression of this marker in DPSCs cells is also high. To justify this conclusion, it can be said that the expression of the β tubulin III marker in DPSCs expresses the potential for higher neuronalization of these cells. In addition, it can be due to the fact that the origin of the DPSCs cells is from the neural crest cells. These results are in line with the results of Miura *et al*., 2003 which proved the pulpal stem cells expressing the β tubulin III marker by using immunocytochemistry and western blotting methods.[20]

Neu N as a special marker for mature nerve cells, was identified in 1992 and can be traced in cell nucleus.[21]

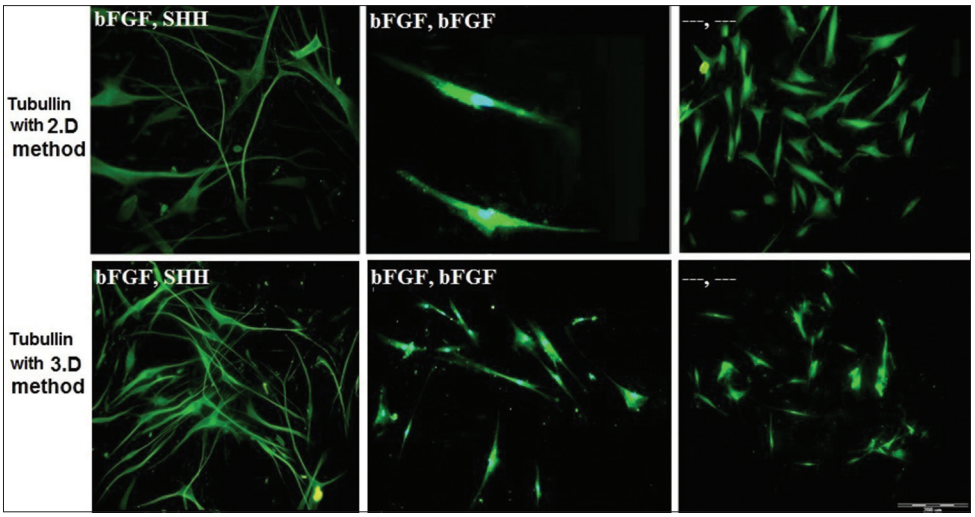

Figure 2: Immunocytochemistry of β tubulin III expression in hDPSCs. In 3D/SHH and in 3D/bFGF groups, the mean percentage of cells which express β tubulin III marker was higher than other groups, Scale bars represent 200 μm. hDPSCs: Human dental pulp stem cells; 3D: Three-dimensional; bFGF: Basic fibroblast growth factor; SHH: Sonic hedgehog.

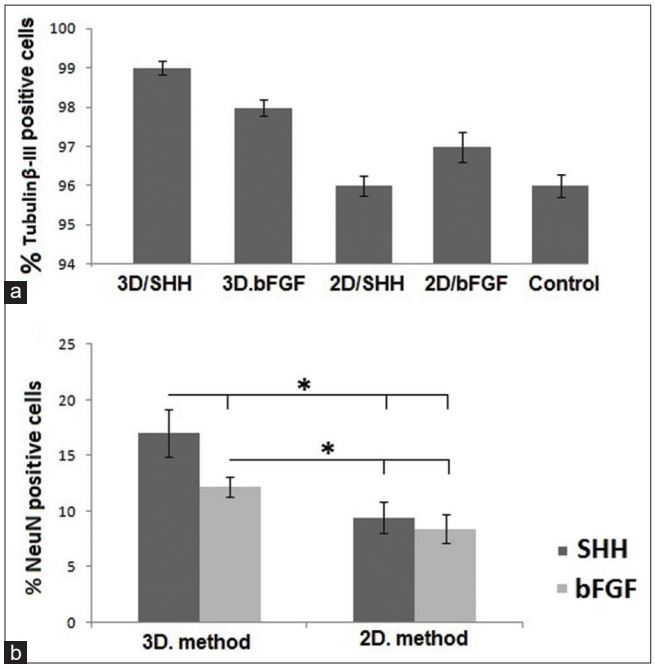

Figure 3: The comparison of β tubulin III and Neu N expression in hDPSCs. (a) The mean percentage of cells which express β tubulin III expression was higher in 3D/SHH and in 3D/bFGF groups than other groups but was not significantly. (b) The mean percentage of cells which express Neu N marker was significantly higher in 3D/SHH and in 3D/bFGF groups than other groups especially in the presence of SHH compared to bFGF (*$P \leq 0.05$). hDPSCs: Human dental pulp stem cells; 3D: Three-dimensional; bFGF: Basic fibroblast growth factor; SHH: Sonic hedgehog.

As can be seen in Figures 3 and 4, Neu N marker is not expressed in DPSCs. This result is consistent with the results of Miura *et al*. study which used immunosuppressive technique proved that the Neu N marker is not expressed in stem cells of human deciduous pulp, but has little expression using Western blotting.[20] In addition, the expression of Neu N marker is significantly higher in differentiated cells within 3D groups than 2D groups. In addition, the percentage of Neu N positive cells is significantly higher in the presence of SHH compared to bFGF. These results may due to attributed to the broad spectrum of SHH performance compared to bFGF, signaling pathway, and the intercellular interaction.

Factor bFGF is a family member of fibroblast growth factors that is found in natural tissues and plays a major role in the formation of blood vessels and in the process of neurogenesis.[22-24] In addition, molecular studies have shown that FGF is a major factor in inhibiting the SHH signaling pathway.[25]

SHH is a member of the Hedgehog protein family and plays a major role in the early stages of neurodevelopment.[26] The results of previous studies have shown that SHH is essential for the formation of the nervous system pattern, neural tube closure, evolution, and axonal conduction.[27,28]

SHH signaling pathway is one of the main pathways in the differentiation of the embryonic neuroectodermal layer and plays an essential role in promoting neuronal differentiation.[29] In addition, one of the most important methods in the process of cellular signaling is juxtacrine signaling. In this cellular messaging method, two adjacent cells communicate directly through specific cell membrane proteins without the presence of any

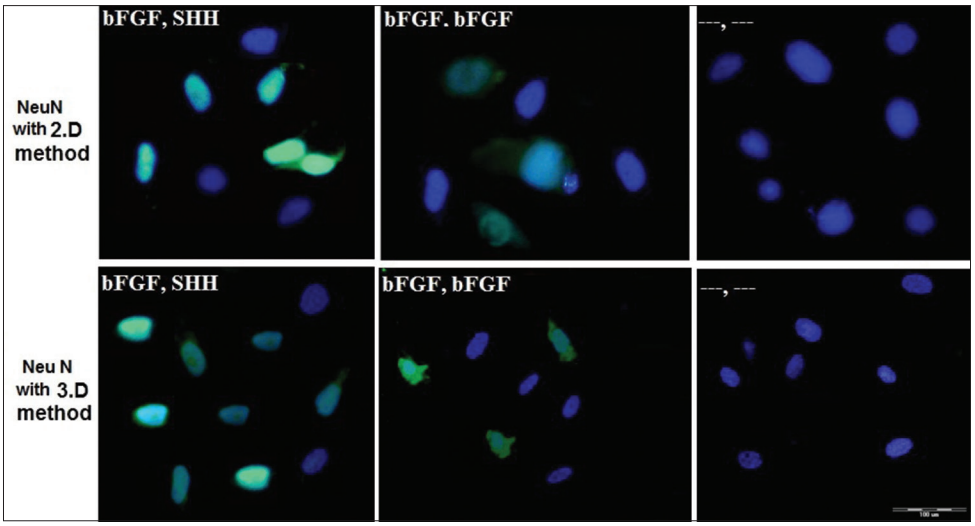

Figure 4: Immunocytochemistry of Neu N expression in hDPSCs. In 3D/SHH and in 3D/bFGF groups, the mean percentage of cells which express Neu N marker was higher than other groups, Scale bars represent 100 μm. hDPSCs: Human dental pulp stem cells; 3D: Three-dimensional; bFGF: Basic fibroblast growth factor; SHH: Sonic hedgehog.

mediating factor. As mentioned in the results, the percentage of cells expressing neural markers in the 3D groups is higher than 2D and control groups. To justify this, it can be said that in the hanging drop method, the stem cells are placed next to each other in the form of a set, and therefore, the conditions are provided for the creation of porous connections between them. Therefore, the intercellular interaction is increased and the cell differentiation process is more completed. The results of previous studies have also shown that in the evolution of the neural tube, perforated connections are formed between neuropithelium cells, and these connections play an important role in advancing the evolution of the nervous system.[30-32]

CONCLUSION

Due to the wide range of SHH functions and the facilitation of intercellular connections in the hanging droop method, it is recommended that the use of hanging drop method and SHH factor can be effective in increasing the efficiency of cell differentiation of dental pulp stem cells into neuron cell-like.

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Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or nonfinancial in this article.

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