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CLINICAL STUDY

Neural derivation of human dental pulp stem cells via neurosphere technique

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ABSTRACT

INTRODUCTION: Human dental pulp stem cells (hDPSCs) are multipotent stem cells providing an autologous noninvasive cell source. The study evaluates the neurogenic potential of hDPSCs using neural growth factor inducers and neurosphere technique.

METHODS: The hDPSCs were differentiated into neurons using neural induction medium containing retinoic acid (RA). Neuroprogenitor cells were evaluated for nestin and NF68 using immunocytochemistry. The mature neuron markers, MAP-2 and β-tubulin, were investigated at the end stage of induction phase.

RESULTS**:**The neuroprogenitor differentiation was confirmed by immunostaining for nestin and NF68 markers. The differentiated neurons were positive for specific neuron markers, namely for MAP-2 and β-tubulin. The results indicated that the neural differentiation medium and neurosphere technique improve the generation of neuroprogenitor cells as well as mature neurons via exhibiting specific neural markers, namely nestin, NF68, MAP-2 and β-tubulin.

CONCLUSION: Our findings highlight the differentiation capacity of hDPSCs via neurosphere technique in the presence of neural inducers for mesenchymal stem cells. It is suggested that the neural differentiation potential of hDPSCs can be exploited as a source of stem cells for therapy of neurodegenerative diseases *(Fig. 5, Ref. 20).* Text in PDF www.elis.sk.

KEY WORDS: mesenchymal stem cells, dental pulp stem cells, neuron, neuroprogenitor cells, multipotent stem cells.

Introduction

Human dental pulp stem cells are multipotent stem cells possibly considered to be a new noninvasive autologous source for MSCs (Gronthos et al, 2000; Sloan et al, 2007). Their origin is similar to that of neural cells. Therefore their neural differentiation capacity can be acceptably exhibited (Huang, 2008; Gronthos et al, 2000).

The hDPSCs can differentiate into various lineages such as adipocytes, osteocytes, neurons and glial cells (Sloan, 2007; Gronthos S et al, 2002). Although the ability of hDPSCs to differentiate into neurons has already been shown, the maturity has not been yet investigated. The aim of this research was to improve the induction technique for *in vitro* transdifferentiation of hDPSCs into mature neurons in the presence of neural inducers.

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The derivation of neuroprogenitor cells was evaluated using the expression of specific neuroprogenitor markers, namely nestin and NF68 as.

The differentiation of hDPSCs has been done previously with neural inducers, but there are no results of it being done via the neurosphere technique. Hence, the goal of our research was to improve the neural induction technique for deriving mature neurons from hDPSCs using neural growth factor inducers and neurosphere technique.

Materials and methods

Culture of hDPSCs

The hDPSCs were isolated from human dental pulp collected from third molar teeth at the dental clinic of Mazandaran University of Medical Sciences. The tissues were digested mechanically and enzymatically and then they were centrifuged. Then, the supernatant was removed and cultured in DMEM/F12 containing 15 % FBS (Gronthos S et al, 2002).

Multipotency of hDPSCs

Osteogenic and adipogenic differentiation of hDPSCs was evaluated via oil red O and alizarin red staining.

Flow cytometry

Isolated cells were examined immunophenotypically for the

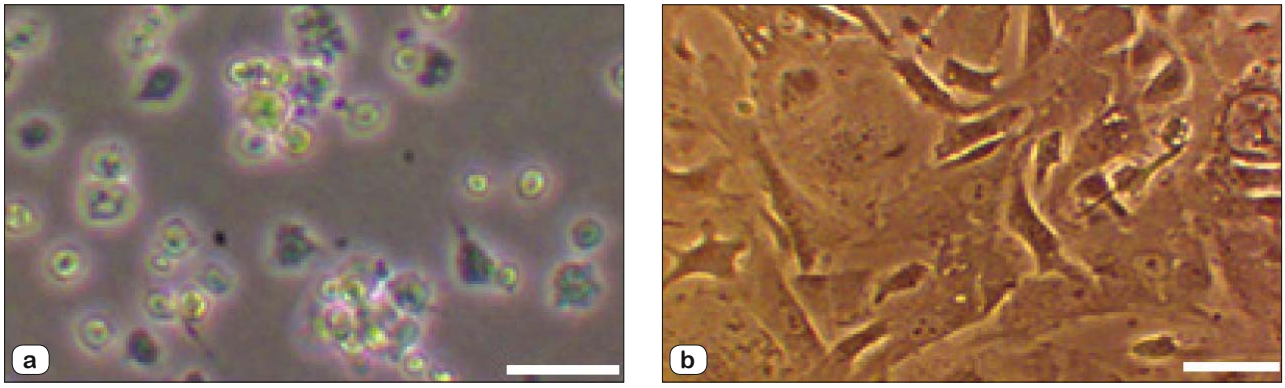

Fig. 1. Phase contrast photography of human dental pulp stem cells after four hours in culture (a). Fibroblastic and spindle morphology of human dental pulp stem cells after fourth passage. Scale bars: 10 µm.

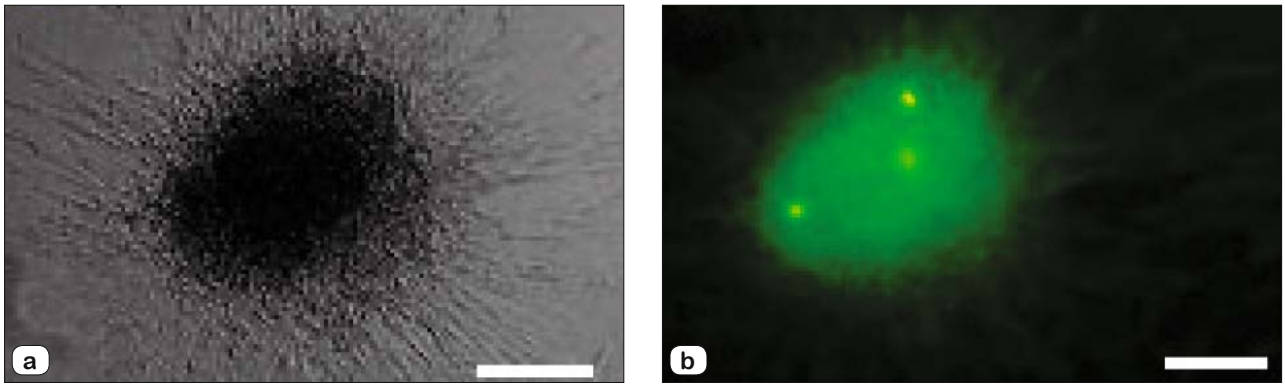

Fig. 2. Phase contrast picture of neurosphere derived from human dental pulp stem cells (a). Neurosphere cells were immunopositive. Scale bars: 500 µm.

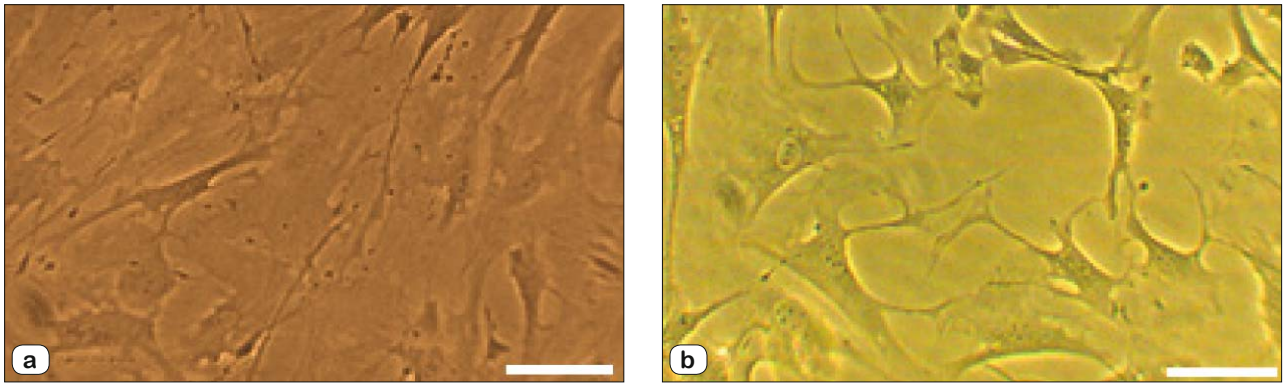

Fig. 3. Neural differentiation of human dental pulp stem cells. Neuroprogenitor cells phenotype (a). Neuron generation at the end stage of induction period (c). Scale bars: 10 µm.

expression of mesenchymal stem cell markers, namely that of CD44, CD90, CD105.

Induction of neurospheres from hDPSCs

Neurospheres were generated from hDPSCs according to the previous method. The cells cultured in four passages, were then trypsinized and plated in non-adherence flasks containing neural stem cells culture medium supplemented with 10 ng/mL of basic fibroblast growth factor, 20 ng/mL of epidermal growth factor, and B27. Neurospheres were formed in the culture after 7–10 days (Fu et al, 2008).

Differentiation of hDPSCs-derived NSCs into mature neurons

Neurospheres were differentiated into neurons according to previous research (Darabi et al, 2015). Neural stem cells were plated onto poly-L-lysine-coated coverslips and in order to enhance their terminal neural differentiation they were cultured for 8–10 days in neurobasal medium containing N2 and B27 supplements.

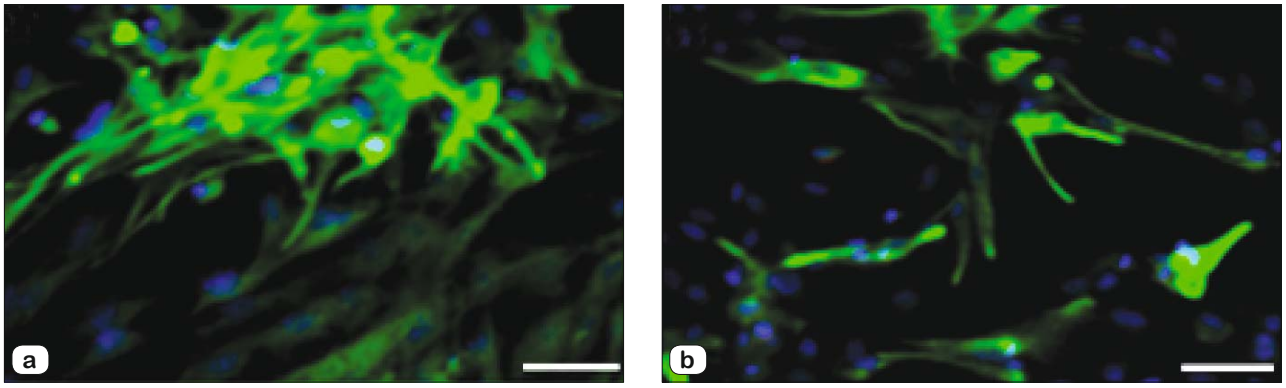

Fig. 4. Immunocytochemistry of differentiated neural stem cells derived from human dental pulp stem cells into neurons. Differentiated cells expressed MAP-2 (a) and tubulin-β (b) proteins. Scale bars: 10 µm.

MTT assay

Methyl thiazolyl tetrazolium (MTT) was used for evaluating the hDPSCs viability.

Immunofluorescent staining

The differentiated cells at the end stage of induction phase were investigated for the expression of neuroepithelial specific markers, namely that of nestin and NF68. Mature neural markers, namely MAP-2 and β-tubulin, were evaluated at the end stage of induction phase. Briefly, the cells were fixed in 4 % paraformaldehyde (pH 7.4) for half an hour at room temperature. Then they were permeabilized with 0.2 % Triton X-100 for 10 min. After washing with PBS they were blocked with 10 % goat serum. Cells were incubated in primary antibodies overnight.

The used primary antibodies were mouse anti-nestin monoclonal antibody (abcam; 1:500) mouse anti-MAP-2 monoclonal antibody (abcam; 1:300), and β-tubulin monoclonal antibody (abcam; 1:200). After washing, the cells were washed and incubated with the secondary antibody, fluorescein isothiocyanate (FITC; 1:1000) for one hour. Cells were mounted with 4,6-diamidino-2-phenylindole (DAPI/PBS; 1:1000). Images were captured with an Olympus phase.

Statistical analysis

SPSS 13.0 software One-way analysis of variance (ANOVA) was used for analyzing the data, and then by Tukey post hoc test ($p < 0.05$ was considered significant).

Results

Specific characteristics of hDPSCs

The hDPSCs were attached, proliferated rapidly and filled the bottom of culture dish. They appeared as spindle shaped or with fibroblastic morphology (Fig. 1). As we showed in previous research, the mesenchymal markers, namely CD 44, CD 90, and CD 105, were detected via flow cytometry technique (Moayeri et al, 2017). Multilineage differentiation of hDPSCs was proved via confirming the osteogenic and adipogenic differentiation (Moayeri et al, 2017).

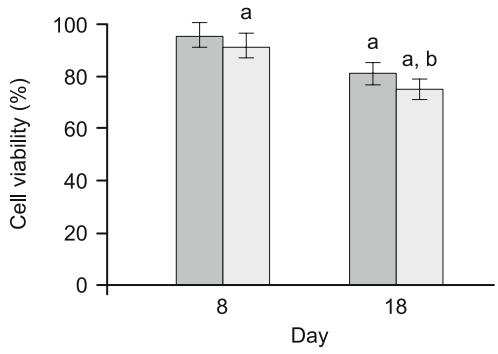

Fig. 5. MTT dates showing viability rates on days 8 and 18 of induction protocol. Significant decrease in control group on day 8 (a). Significant decrease in control group on day 18 (b).

Derivation of NSCs from hDPSCs

Following the fourth passage, hDPSCs were trypsinized, and cultured in non-adherence flasks containing neural stem cells medium supplemented with bFGF and EGF. Neurospheres were formed after 7–10 days (Fig. 2). The derivation of NSCs from hDPSCs was accompanied with a wide range of morphological changes.

Derivation of mature neuron from NSCs

NSCs transformed to mature neurons with branched morphology after induction protocol (Fig. 3).

Immunostaining data proved the expression of neuroepithelial markers including nestin in NSCs (Fig. 2). The differentiated mature neurons were immunopositive to specific markers as e.g. MAP-2 and β-tubulin (Fig. 4).

Viability of hDPSCs during induction steps

The survival rate of cells at the first stage of induction for experiment and control were evaluated via MTT test that showed 91.58 ± 0.33 % and 95.72 ± 0.36 %, respectively. Also, the viability rate showed a significant decrease between case and control groups at the end of protocol ($p < 0.05$) (Fig. 5).

Discussion

Mesenchymal stem cells have an ability to differentiate into a neural phenotype after being exposed to neural inducers (Delaviz et al, 2014, Ghasemi et al, 2014, Le Blanc et al, 2005, Nazm Bojnordi et al, 2015). The hDPSCs arise from the neural crest and possibly are considered to be an appropriate cell source for neuronal generation usable in the treatment of neurodegenerative diseases (Yalvac et.al.2009, Huang et.al.2008).

Recent research showed that mesenchymal stem cells derived from bone marrow can convert into NSCs (Sanchez-Ramos et al, 2000, Ping et al, 2015, Darabi et al, 2013). We have used these protocols for hDPSCs which represent a promising therapeutic approach in neuroregenerative medicine. By using MSCs derived from human teeth, we have proved that under specific culture conditions, hDPSCs have the ability to form NSCs . Theses NSCs can differentiate into neurons and exhibit mature neural markers detectable via immunocytochemistry technique.

The potential of hDPSCs to differentiate into neurons has already been proven in previous studies, but the maturity of neurons has not yet been evaluated (Chang et al, 2014, Chun et al, 2016, Ghasemi et al, 2017). Therefore, we have designed an improved modification of induction technique for *in vitro* differentiation of hDPSCs into mature neurons.

NSCs-derived hDPSCs were transformed from the proliferation stage into the differentiation phase, they exhibited the morphology of neuroprogenitor cells and were immunopositive to nestin and NF68 markers. Our data are similar to those in previous reports in the sense that the neural differentiation of mesenchymal stem cells is associated with nestin and NF68 as neuroprogenitor markers (Haratizadeh et al, 2016).

Culturing the cells in neural stem cells medium supplemented with bFGF and EGF completed the neural differentiation and exhibited specific neural markers, namely MAP-2 and β-tubulin.

The generation of neural phenotype from hDPSCs which has been proved in this research is the basis for a proposition that hDPSCs are an appropriate alternative source for improving the treatment of neural damage in neuroregenerative medicine. Nevertheless, more investigation is needed to approve the functionality of mature neuron derived from hDPSCs.

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