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EXPERIMENTAL STUDY

Enhanced yield of cholinergic neurons from induced pluripotent stem cells (iPSC): A two-step induction protocol

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ABSTRACT

BACKGROUND: Cholinergic neurons, a type of neurons found in central nervous system, play a vital role in muscle movement and activities. Cholinergic neurons degeneration is the main pathological symptom of neurodegenerative diseases. Among a variety of stem cells, iPSCs have emerged as a promising candidate for transplantation to improve the repair of neuronal lesion sites. However, the establishment of an appropriate induction method to yield large numbers of cholinergic neurons has yet to be determined. Here, we studied the differentiation potential of iPSCs to generate cholinergic neurons by developing a new optimized differentiation protocol.

METHODS: The iPSCs were harvested on 6-well matrigel-coated plate and incubated with serum-free DMEM/F12 with 2 % B27 supplement, 20 ng/ml the basic fibroblast growth factor and 20 ng/ml epidermal growth factor for 48 hours. Then, the pre-induced cells were treated in neuronal induction medium supplemented with all-trans retinoic acid, sonic hedgehog, 100 ng/ml glial-derived neurotrophic factor and 200 ng/ml brain-derived neurotrophic factor for 7 days. Cell viability during induction stages was tested by MTT assay. Differentiated cells were evaluated with crystal violet staining, immunocytochemistry and real-time PCR.

RESULTS: Our results showed that the survival rate of iPSCs leveled out and was similar to that in the control group following the differentiation process. Immunochemistry results revealed that the expression of ChAT was observed in cells in both pre-induction and induction stages with a significantly higher expression level at the induction stage as compared to the pre-induction stage. However, none of these markers was expressed in the iPSCs. Cresyl violet staining confirmed the neuronal phenotype of differentiated cells. The induction group significantly expressed the higher levels of Islet1, Olig2 and HB9, whereas pluripotency markers including those of Oct4 and Nestin plunged.

CONCLUSION: Our investigation represents a highly efficient protocol for iPSCs differentiation toward cholinergic neurons which could be used for further preclinical transplantation studies *(Tab. 1, Fig. 5, Ref. 35)*. Text in PDF *www.elis.sk*

KEY WORDS: induced pluripotent stem cells, cholinergic neurons, neurotrophic factors, induction protocol, preclinical transplantation.

Introduction

Cholinergic motor neurons, a type of central nerve cells, are specified to transport electric signals between the brain and muscles and bones. They are anatomically located in the brain and the ventral areas of the spinal cord, and together with muscles contribute to regulating the movement functions such as breathing (1–3). Under pathological CNS conditions and through complex processes, the loss of motor neurons occurs, leading to motor neuron degeneration and movement deficits. Motor neuron dysfunction is associated with neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), spinal and bulbar muscular atrophy (SBMA) and even Alzheimer disease (AD) (2, 4). Even though, some drugs have been projected to alleviate symptoms and improve neurological diseases, these therapeutic approaches are poorly effective to treat the loss of neurons. Difficulties such as low capability of CNS for self-regeneration and self-restoration are the other side of challenges which should not be neglected (4–7). Therefore, the precise treatment of neurological disorders is remained to be solved (5, 6). Over recent years, stem cell therapy has been proposed as an alternative approach with the aim of cell replacement at the injury site (5). So far, a variety of stem cells from different sources and origin has been widely studied in the field of neurodegenerative diseases and some

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of them have been successfully applied clinically. Regarding to motor neurons-related disorders, ESCs are among the most typical stem cells that have been used in preclinical disease models. Meanwhile, there are some hurdles including allogeneic immune rejection, ethical concerns and potential tumor formation which limit the clinical application of these cells (8, 9). In addition, mesenchymal stem cells which are privileged for their easily accessible sources without ethical concerns, might have some drawbacks such as insufficient neural differentiation capacity and immune rejection (10). Over the past decade, the differentiation potential of novel reprogrammed somatic cells, induced pluripotent stem cells (iPSCs), has been exclusively studied (6, 11). The iPSCs which are first established by Yamanaka's laboratory in 2006, can be derived from patient's own somatic cells blood, fibroblasts and urine cells and are capable of differentiation into all cell types of three germ layers, i.e., ectoderm, mesoderm and endoderm (12–15). Despite being identical to ESCs in differentiation ability, they do not face the ethical and immunogenic issues attributed to ESCs (9, 10). Since then, iPSCs have been broadly employed in various biomedical and tissue engineering fields from understanding of the pathological mechanism of motor neuron disorders using *in vivo* disease models and *in vitro* environment to drug screening and even in clinical treatment of stem cells transplantation (10, 16, 17). Some studies have shown that iPSCs could be differentiated into dopaminergic neuron-like cells (18–22). The differentiation of iPSCs toward cholinergic neurons from various protocols has been reported as well (23–26). However, there are still major challenges in the way of developing an appropriate induction protocol, such as variable efficiency, low purity and number of differentiated cells as well as the long period of induction stages, which is a great problem. Therefore, setting up a simple, highly efficient *in vitro* protocol is required to easily differentiate iPSCs into desired neural cells for transplantation. In the present study, we aimed to optimize the *in vitro* environment in association with physiological condition to achieve highly rational cholinergic-like cells. We established a well-optimized two-step protocol for differentiating iPSCs to cholinergic neurons.

Materials and methods

Induced pluripotent stem cells culture

The gifted human iPSCs (hiPSCs) line donated by Prof. Masoud Soleimani (from Stem Cells Technology Research Center, Tehran, Iran) was used in this study. In order to expand the cells, they were cultured on a mouse embryonic fibroblast (MEF) feeder cell layer in medium containing DMEM/F12 supplemented with 20 % serum, 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, 10 ng/mL basic fibroblast growth factor (bFGF), and 0.1 mmol/L nonessential amino acids (all from Invitrogen). The cells were incubated at 37 °C with 5 % CO_2 and 95 % humidity. Every two days, the medium was removed, and fresh medium was added. After reaching a 90 % confluency, the cells were enzymatically detached and passaged onto another MEF layer using 0.1 % Collagenase type IV (Invitrogen) (27).

To increase the differentiation efficiency, the iPSCs embryonic bodies (EBs) production was applied. Briefly, after detaching of the iPSCs colony, they were transferred to the nontreated plates and incubated with EB formation medium for 4–5 days.

Induction of iPSCs differentiation

In order to induce differentiation, iPSCs were cultured on a 6-well matrigel-coated plate (BD

Biosciences) containing serum-free DMEM/F12 with 2 % B27 supplement (Invitrogen, Scotland) and 20 ng/ml the basic fibroblast growth factor (bFGF: Invitrogen, Scotland) and 20 ng/ml epidermal growth factor (EGF: Invitrogen, Scotland) for 48 hours (pre-induction stage). Then, pre-induced cells were treated in neuronal induction medium supplemented with all-trans retinoic acid (RA; 0.1 μM, Sigma), sonic hedgehog (Shh; 1 μg/ml, R&D Systems), 100 ng/ml glial-derived neurotrophic factor (GDNF, Invitrogen, Paisley, Scotland) and 200 ng/ml brain-derived neurotrophic factor (BDNF, Invitrogen, Paisley, Scotland) for 7 days (induction stage). Morphological assessment of differentiating cells was analyzed in different groups by inverted microscope (Nikon, Eclipse-TS100).

MTT assay

Cell viability was evaluated using the MTT colorimetric assay after every 3 days of induction, i.e., on days 3, 6 and 9 (28). Briefly, cells were seeded at a density of 5,000 cells/well on

96-well plates and incubated at 37 °C, overnight. until a comparable confluence to the ATR FTIR experiment was observed in the wells. The medium was then removed and incubated with 50 μl MTT solution (sigma Aldrich, 1mg/ml PBS) for 3 h at 37 °C in the dark. Then, the supernatant was discarded and 50 μl/well dimethyl sulphoxide (DMSO, Sigma-Aldrich) was added. Plates were shaken for 10 min to dissolve formazan crystals. The absorbance was measured at 570 nm by a Cytofluor 4000 plate reader (PerSeptive Biosystems, USA). All tests were conducted in triplicate (29).

Tab. 1. Primer sequences used in real-time PCR.

Gene	Significance	Forward primer (5´- 3´)	Backward primer (5´- 3´)	size (bp)
Oct4	Stemness marker	TATGCAAATCGGAGACCTG	AAGCTGATTGGCGATGTGAG	143
Nestin	Neuroprogenitor marker	AAGAGAGCATAGAGGCAGTAA	GAGATTTCAGTGTTTCCAGGT	93
NF-M	Differentiating neuronal markers	GACGGCGCTGAAGGAAATC	CTTGGCGGAGCGGATGGCCT	142
Islet1	Precursor motor neuron marker	CACACTCGGATGACTCTGG	CTTGCGGACCTGGTATGC	99
Olig2	Progenitor motor neuron marker	CCAGCAACAATTAACTTAGG	CTTAGAAGAGACGCAGAG	155
HB9	Mature motor neuron marker	ATGAGGATGAGGATGATGAAGAAG	CAGATGAGCAATCGGATGAGG	138
GAPDH	Internal control	GTTGTCTCCTGCGACTTCA	CTTAGAAGAGACGCAGAG	190

Cresyl violet stain

The cellular Nissl bodies can be evaluated by means of cresyl violet specific staining that was performed on the final day of induction process. to end this, on days 3, 6 and 9 of induction, cells were fixed with 4 % PFA for 20 minutes and washed with PBS three times. Then, cells were stained with 1 % cresyl violet stain (Merck Germany) for 2–3 minutes and were morphologically observed under a light microscope. Cells with pink Nissl bodies are considered as positive cells.

Real-time PCR

Total cellular RNA was extracted at various differentiation stages by trizol reagent (Invitrogen Life Technologies) according to the manufacturers' instructions and the purity and concentration of RNA was determined using spectrophotometer at the absorbance A260/A280. Afterward, 5 µg purified RNA template reverse transcribed into cDNA by oligo dT primer, reverse transcriptase (Fermentas) and random primers (Fermentas) under standard conditions in accordance with the manufacturers' instructions (30). Quantitative real-time RT-PCR (Corbett Research, Qiagen, Germany) was carried out to confirm the differentiation by evaluating the expression level of specific genes including *Oct4, Nestin, NF-M, NF-H, Islet1, Olig2, HB9 and GAPDH.* Primer sequences are shown in Table 1. Briefly, for each reaction, 1 µg of cDNA was amplified using qPCR reaction solution components, 10 µL SYBR green, 20 µL of deionized distilled water (ddH2O) and 300 nM of forward and reverse primers (28, 31). Amplification was performed at following thermal condition setting: 95 °C for 15 min for initial denaturation was followed by 45 cycles of denaturation at 94 °C for 15 seconds, 55 °C or 62 °C for 30 seconds for annealing and elongation at 72 °C for 45 seconds for 45 cycles.

Immunocytochemistry

Cells were washed with PBS for 5 min, and then fixed with 4 % PFA for 15 min at room temperature (RT). Then, the supernatant was discarded and cells were rinsed with PBS, treated with 1 % Triton X-100 (Sigma-Aldrich) for 30 min at RT and blocked with 2 % BSA (bovine serum albumin, Sigma-Aldrich) for 45 min. Cells were incubated with primary antibodies including rabbit polyclonal anti human Nestin (ab92391, Abcam system, UK) and ChAT (ab137349) separately on each well for 1h, washed again with PBS (3x), which was followed by incubation with secondary antibody (Goat anti-Rabbit FITC, ab97050, abcam system, UK) for 1h at 37 °C. The cells were then rinsed twice in PBS for 15 min and counterstained with propidium iodine to visualize the nuclei, and counted under the fluorescent microscopy (Nikon, Eclipse-TE600, Japan). The percentage of immunoreactive cells was randomly calculated. Data were achieved from 3 independent experiments (32).

Statistical analysis

All data were statistically analyzed by means of SPPSS 16 software. One-way ANOVA and Chi Square tests were applied to compare variables between groups. The results were expressed as means ± S.D. p values were considered significant when $p < 0.01$ and $p < 0.05$.

Results

Characterization of iPSCs

Human iPSCs were cultured on the inactivated mouse embryonic fibroblast as a feeder layer and its colonies were formed 1 week after cell seeding (Fig. 1A). Then, to begin the differentiation, the iPSC colonies were detached by collagenase IV and transferred to the low-attachment culture plate under the EB formation medium. After 4–5 days, iPSC-derived EBs were formed and observed using an inverted microscope (Fig. 1B).

Survival rate of iPSCs

To test the viability of cells during induction process, MTT assay was performed and the viability of cells on days 3, 6 and 9 of induction was compared with that of the control group. Our findings demonstrated that there were no differences between the two groups in the percentage of live cells for 9 days (Fig. 2).

Cytological studies of differentiated cells

Morphological changes of iPSCs during differentiation were determined using cresyl fast violet staining. As it has been demonstrated in Figure 3D, the cells were reacting positively to cresyl fast violet which showed that the induced cells had the neural phenotype.

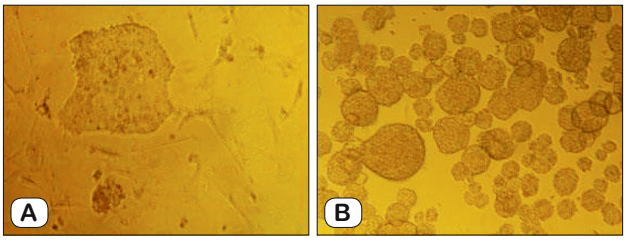

Fig. 1. Photographs of human induced pluripotent stem cells (iPSCs) colonies while cultured on inactivated mouse embryonic fibroblast (A) and human iPSCs embryoid bodies (EB) after 4 to 5 days culturing under iPSCs EB medium (B).

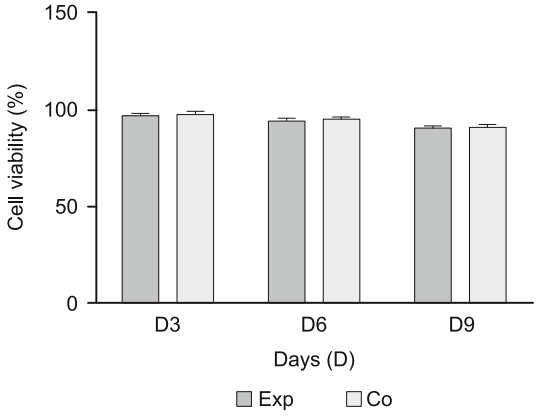

Fig. 2. Viability percentage of human induced pluripotent stem cells (iPSC) was evaluated using MTT test at 3, 6 and 9 days. Error bars represent the standard deviation.

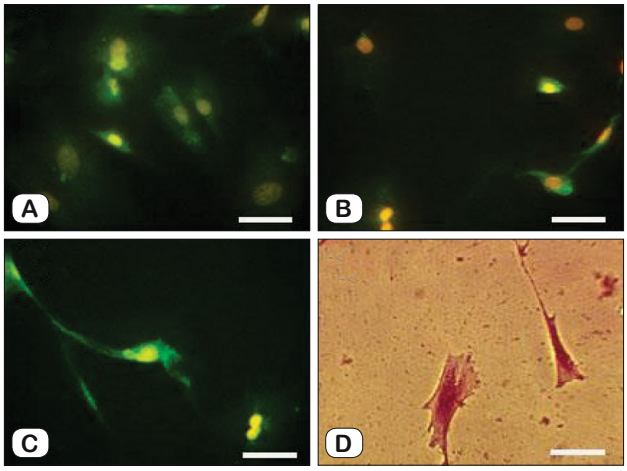

Fig. 3. Photomicrographs of induced pluripotent stem cells (iPSCs) for different markers during differentiation into cholinergic phenotype. A and B represented Nestin in pre-induction and induction stages. Part C demonstrated ChAT immunostaning in induction stage and D represented crystal violet specific staining (Scale bars: 25 µm).

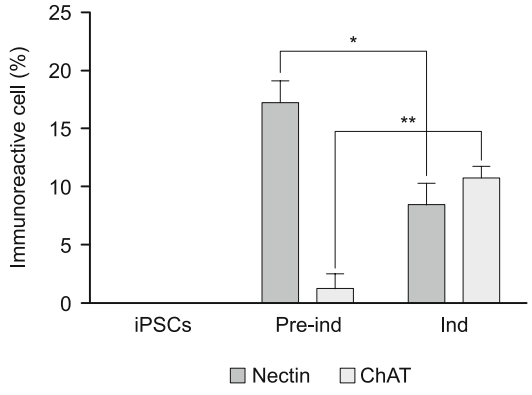

Fig. 4. Histogram of the percentage of induced pluripotent stem cells (iPSCs) differentiated into cholinergic neurons on day 2 (pre-induction stage, Pre-ind) and 9 (induction stage, Ind). Values are expressed as mean ± SD (n = 3). *indicates the statistical significance of Nestin expression between day 2 and 7 ($p < 0.05$), **indicates the statistical significance of ChAT expression between day 2 and 9 ($p < 0.01$).

Immunocytochemistry staining

Immunocytochemistry staining of the Nestin and ChAT markers were performed at different stages (Fig. 3). The iPSCs expressed Nestin at pre-induced and induced stages. However, after 7 days, the expression of Nestin in cells significantly decreased during the induction stage ($p < 0.05$). The expression of ChAT was observed in cells of both pre-induction and induction stages with a significantly higher expression level ($p < 0.01$) at the induced stage compared to the pre-induced stage (Fig. 4). However, none of these markers was expressed in the iPSCs.

Real Time-PCR

The expression level of *OCT4, Nestin, NF-M, Islet1, Olig2* and *HB9* genes were analyzed using qRT-PCR. As it has been shown

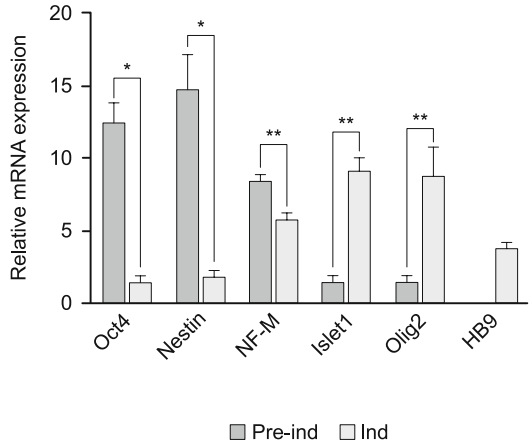

Fig. 5. Real-time PCR analysis for in vitro differentiation of neural and cholinergic markers of induced pluripotent stem cells (iPSCs) on day 2 (pre-induction stage, Pre-ind) and 9 (induction stage, Ind) (n = 3 biological samples, mean ± SD; ** $p < 0.01$, * $p < 0.05$).

in Figure 5, before the induction process, the expression level of *Oct4, Nestin and NF-M* were significantly higher in comparison to those of *Islet1* and *Oligo2* genes. Interestingly, after induction, there was a dramatically decrease in the expression of *Oct4, NF-M* and nearly *NF-M*, whereas those of *Islet1* and *Olig2* significantly increased. The *HB9* level was also observed in differentiated cells after induction.

Discussion

Neural regeneration is one of the major challenges in the treatment of neurodegenerative diseases. In spite of many investigations, the regeneration has remained unsolved due to some restrictions on having access to these cells (7, 10, 26, 33). Stem cell therapy has been widely considered as an effective approach, which enhanced in generating a range of preclinical and and clinical trials dealing with neural cells for cell transplantation in neurodegenerative diseases. It has been confirmed that iPSCs offer new insights into the study of neurological diseases (7, 26, 28, 34, 35). Until now, several methods have been established for evaluating the differentiation process of iPSCs into cholinergic cells. However, most of them have been less effective such as to be applied in the clinical setting (1, 15, 30). In our study, we optimized a method by which iPSCs could effectively differentiate into cholinergic neuron-like cells. In this work, we divided iPSCs into 2 groups including pre-induced iPSCs and induced iPSCs which was followed by growth factor treatment . Previously, βME have been shown to have a potential to promote differentiation potential of bone marrow stem cells (BMSCs) into neural cells and human mesenchymal stem cells into osteoblast cells. Nonetheless, there is no report whether it can affect the differentiation of iPSCs into cholinergic motor neurons. Moreover, RA is one of the major factors that regulate neural cells, differentiation,

growth and survival in the nervous system. There is also a major role of neurotrophin which is directly involved in promoting the peripheral nerve regeneration following injury (6, 14, 24, 32, 35). Previous studies have reported that exposure of iPSCs to nerve growth factor (NGF) promotes the differentiation of these cells into various types of neurons such as basal forebrain-like cholinergic neurons. In this study, we successfully showed that cocktail growth factor effectively induced cholinergic neuronal phenotype in iPSCs during the differentiation process. According to our data from real-time PCR, the expression level of *Olig2* and *Islet1* in induced iPSCs increased.

More strikingly, our findings showed that upregulation of *Olig2* and *Islet1* during differentiation were accompanied with a low expression level of *NF-M, Oct4* and *Nestin* in this group, whereas, in pre-induced iPSCs, the expression level of *Nestin* and *Oct4 was much higher*. Cholinergic motor neurons are a family class of extrapharyngeal cholinergic neurons and are identified with ventral markers Olig2 and Islet1. Olig2 or oligodendrocyte-specific bHLH transcription factor plays an important role in the differentiation potential of cell lineage toward motor neuron. Lack of Olig2 leads to deficits in the differentiation program and directs cells into other neuron cell types. Islet1 is another transcription factor which is crucial in survival and differentiation of both sensory and motor neurons.

On the other hand, apart from chemical reagent used for induction program, it is worth mentioning that the duration of induction process was successfully optimized.

Our investigation represents a highly efficient protocol for iPSCs differentiation toward cholinergic neurons which could be used in further preclinical transplantation studies.

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