ORIGINAL ARTICLE

MEVASTATIN: A NOVEL AGENT FORMING DOPAMINERGIC NEURONS FROM HUMAN MESENCHYMAL STEM CELLS

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Background: Human umbilical cord derived mesenchymal stem cell (hUMSCs) are in experimental phase for treatment of various neurological disorders. Differentiating these cells into neurons is expensive and painstaking. Mevastatin a commonly used antihyperlipidemic agent is known for its neuroprotective effect. We investigated the differentiation response of human umbilical cord derived mesenchymal stem cell (hUMSCs) to Mevastatin. **Methods:** In this dose-response experimental study, hUMSCs were isolated and characterized for presence of specific stem cells markers (cd90, cd73, cd105, oct4) by conventional PCR and Immunocytochemistry (Vimentin, cd24, cd90). Then, the cytotoxicity of the compound (Mevastatin) was analyzed to select the best working concentration for neuronal differentiation. Cells were grown in the presence of the least cytotoxic concentration for 2 weeks. The differentiated cells were analyzed for morphological changes and neuron specific markers by qPCR and Immunocytochemistry. **Results:** hUMSCs derived dopaminergic neurons to be similar to those found in the human midbrain based on cell type and results showed positive expression of dopaminergic neuron specific genes (TH, NURR1, LMAX1). Positive expression of TH was observed in the differentiated cells. Additionally, the differentiated cells lost the expression of stem cells specific marker cd24, which was evident in the control cells. **Conclusion:** Mevastatin can differentiate hUMSCs into dopaminergic neuron like cells at 1 µM concentration. Using Mevastatin (when required) in those dyslipidemic patients who have increased susceptibility to Parkinson's disease is suggested. However, further research in this direction is recommended.

Keywords: Cell-therapy in Parkinsonism, Neurodifferentiation by Mevastatin, Dopaminergic neuron formation

Pak J Physiol 2021;17(4):3–7

INTRODUCTION

Parkinson's disease (PD) is a neurodegenerative progressive disorder affecting millions of people worldwide. It is characterized by typical movement disorder including rigidity, tremors and bradykinesia.[1] It is identified pathogenically by, the presence of Lewy bodies made up of misfolded α-syn[2] protein.[2]

Current treatments of Parkinson's disease (PD) include levodopa, monoamine oxidase type B (MAOB) inhibitors, and catechol-o-methyl-transferases (COMT) that help in improving few symptoms of the disease. However, with continuous use patients develop drug resistance and eventually other modalities need to be used. A common such method is deep brain stimulation (DBS). This has limited benefit in reduction of symptoms and can be used only in some specific patients.[3]

In PD Gene therapy has been considered as hopeful treatment approach. The aim of gene therapy is to modify the expression of neurons in basal ganglia that have degenerated with resultant decreased release of dopamine.[4] Genetic modifications are in the experimental phase and require extensive testing before these can be introduced in humans.[5,6]

Cell-based therapies have attracted attention of the researchers as being potentially feasible for neurodegenerative diseases. It encompasses derivation of specific neuronal cells and consequent transplantation into affected parts of the neuronal system. Different cell types have been used for differentiation of dopaminergic neurons and subsequent transplantation into patients with PD.[7] These include embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs) etc. ESCs have a potential to differentiate into 3 three primary germ layers. However, their use is restricted due to ethical issues and high risk of teratoma formation after transplantation.[8] iPSCs on the other hand are very important tools for drug development, regenerative medicine and diseases modelling. Furthermore, they are considered superior to ESCs as a cell source for PD replacement, including the capability to use patient's peculiar cells and consequently reduce the necessity for immuno-suppression. However, their

use could be limited due to their high risk of teratoma formation after transplantation.[8]

Adult stem cells are obtained from all tissues of the three germ layers, for example MSCs can be derived from human amnion epithelial cells. They are easy to separate, have more differentiation potential with fewer ethical problems.[9,10] MSCs release the soluble factors that are significant for cell existence and proliferation.[11] Different small molecules have been used to differentiate hUMSCs into neurons. These include Valproic acid fibroblast growth factor and Forskolin.[12] It was observed that statin molecules are effective in both neuronal differentiation and mid brain neuron specification.[12] Various statin molecules have been implicated to cause similar differentiation, however Mevastatin has been shown to be superior in this effect to others.[13]

This study was designed to investigate the differentiation potential hUMSCs into dopaminergic neurons using Mevastatin.

METHODOLOGY

This was a dose response in-vitro experimental study carried out at Ziauddin University from August 2019 to February 2020 after approval from the Ethics Review Committee of Ziauddin University.

Umbilical cords were obtained from babies of primigravida mothers between 18 to 30 years of age with no known comorbid and pregnancy related complications.

The cord was washed numerous times with purified PBS solution and cut into 3 mm thick parts. Cord tissues were minced with scissors and processed with Trypsin 10X act as digest. The isolated cells there were plated in DMEM (Life Technologies) supplemented with 10% foetal bovine serum (FBS), and 100 mg/mL streptomycin 50 mg/mL penicillin (Life Technologies) at 37 ºC in a moistened 5% CO_2 incubator.

RNA was isolated from control hUMSCs with Trizol reagent according to the optimized protocol. The isolated RNA was stored in nucleus free water (Life Technologies) at -80 ºC.

RNA was quantified by using multi scan skey spectrophotometer and the kit revert aid first stand cDNA synthesis kit (M/S Thermo Scientific) was used for one microgram RNA reverse transcription according to manufacturer's protocol.

The cDNA was either used directly for PCR or kept at -20 ºC till further usage.

For Primer designing we used the primer3 design program at http://frodo.wi.mit.edu/primer3/, and bought from Pericon.

q-PCR of hUMSCs cells were used for the expression of stem cells markers (CD73, CD 105, CD 133, Oct-4) GAPDH was used as an internal standard.

Then PCR products were evaluated by gel electrophoresis. The Gel documentation system was used to study the gel.

hUMSCs cells (~10,000 cells) were cultured in 24 well plates, processed according to standard protocol and viewed using an inverted fluorescent microscope.

hUMSCs were treated with different concentration such as (1 µM, 5 µM, 10 µM, 50 µM, 100 µM) of Mevastatin and morphology of the cells was observed at different times intervals 24, 48, and 72 hours with performing the following steps.

To evaluate cytotoxicity in cells after treatment with different concentrations of Mevastatin, trypan blue exclusion (Life Technologies) procedure was performed. Cells were cultured in 24-well plate and incubated with different concentrations of Mevastatin (1 µM, 5 µM, 10 µM, 50 µM and, 100 µM)[12] cytotoxicity was observed at different time interval.

Live and dead cells were counted separately to calculate the cytotoxicity according to the formula:

$$\text{Viable cells (\%)} = \frac{\text{Total No. of viable cells/mL of aliquot}}{\text{Total No. of cells/mL of aliquot}} \times 100$$

When live cells reached 70% to 80% of confluency, by using 1 µM least cytotoxic concentration of Mevastatin was used for the differentiation induction of hUMSCs and media was replaced after each 3rd day followed by morphological and gene expression analysis of the differentiated cells after 2 weeks.

All the groups were analysed for morphological changes by Floid Cell Imaging System and compared with untreated control and images were captured at 40× magnification.

The treated cells RNA was isolated by using Trizol and Revert Aid cDNA synthesis Kit was used for reverse transcription of RNA. Expression of neuron specific markers (TH, NURR1 and LMXa1) were examined by real time-PCR. Following completion of 40 cycles, CT values were acquired and used to determine the result of relative fold chain using the formula:

$$\Delta Ct = Ct\,(Gene) - Ct\,(GAPDH)$$

$$\Delta\Delta Ct = \Delta Ct\,(Sample) - \Delta Ct\,(Control)$$

$$\text{Fold change} = 2^{-(\Delta\Delta Ct)}$$

Differentiated cells (~10,000 cells) were cultured in 24 well plates by adding 1 mL of cell suspension (1× cells). The cells were labelled against primary antibody TH, LMXIA, NURR1 and

CD24 was used as stem cell marker, further procedure had been processed according to standard protocol and viewed using an inverted fluorescent microscope.

Statistical analysis was carried out using SPSS-20. Quantitative variables were calculated as percentages. Student's *t*-test was carried out as a measure of difference and a $p<0.05$ was considered significant.

RESULTS

After propagation, characterization of hUMSCs was done by morphological examination, gene expression analysis (OCT 4, CD105, CD73) using GAPDH gene as internal standard and protein expression analysis (Actin, Vimentin, CD24).

The hUMSCs were then treated with different concentrations of Mevastatin (1, 5, 10, 50 and 100 µM). The cells were analyzed in the following steps:

No cytotoxicity was observed at the concentration of 1 µM at 72 hours. However, we found that cells showed high cytotoxicity and cell death along with no differentiation (neuron morphology) under our observation at 5 µM, 10 µM, 50 µM, 100 µM at 24 and 48 hours respectively. (Figure-1).

Cytotoxicity was calculated using the formula which was mention above. Our results showed that the cell viability at 1 µM was around 93.89% which was highest when compared to other concentration.

Morphological examination of differentiated hUMSCs was carried out 2 weeks after treatment with Mevastatin using inverted microscopy. Dopaminergic-neuron-like cells were found after treatment with 1 µM concentration of Mevastatin at 72 hours. Cells showed multipolar shaped neuron formation. Hence 1 µM concentration of Mevastatin was considered as least cytotoxic and used for differentiation induction. (Figure-2).

Genes expression analysis of differentiated specific genes such as TH, NURR1, LMXIA were determined by using q-PCR. GAPDH was taken as positive control. Our results showed that expressions were significantly increased ($p<0.05$) after 1 µM Mevastatin treatment as compared to control. (Figure-3).

Basal level of Actin, CD24, and TH proteins were analysed in the treated cells by direct immunofluorescence. The cells of control and treated showed positive expression of actin while CD 24 negative expression in treated cells, Tyrosine protein highly expressed in treated cells. (Figure-4).

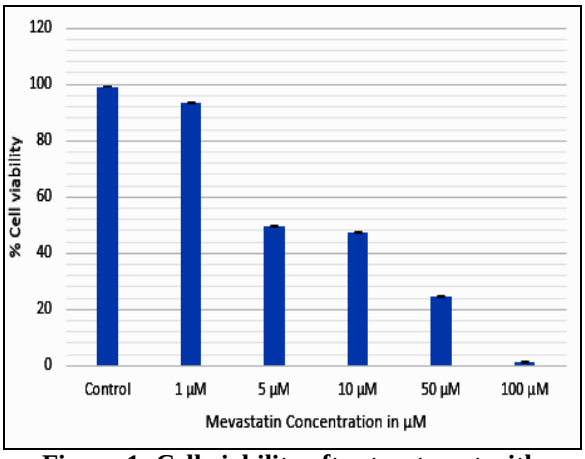

Figure-1: Cell viability after treatment with Mevastatin

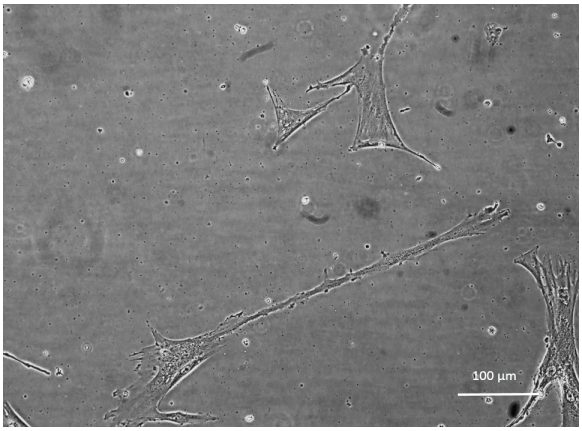

Figure-2: Differentiated hUMSCs were observed dopaminergic neuron like cells after the treatment of 1 µM concentration of Mevastatin at 72 hours after 2 week. Cell showed multipolar in shaped, analyzed under inverted microscopy at 10× magnification.

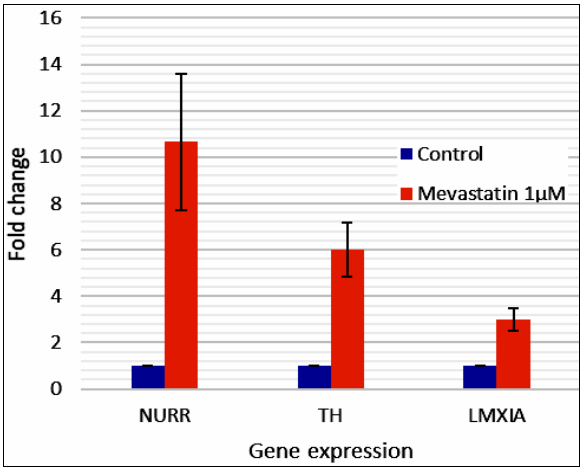

Figure-3: Expression of neuron specific genes by qPCR

Combined graphical presentation of all genes expression were significantly increased ($p<0.05$) in the Mevastatin treatment groups compared to control

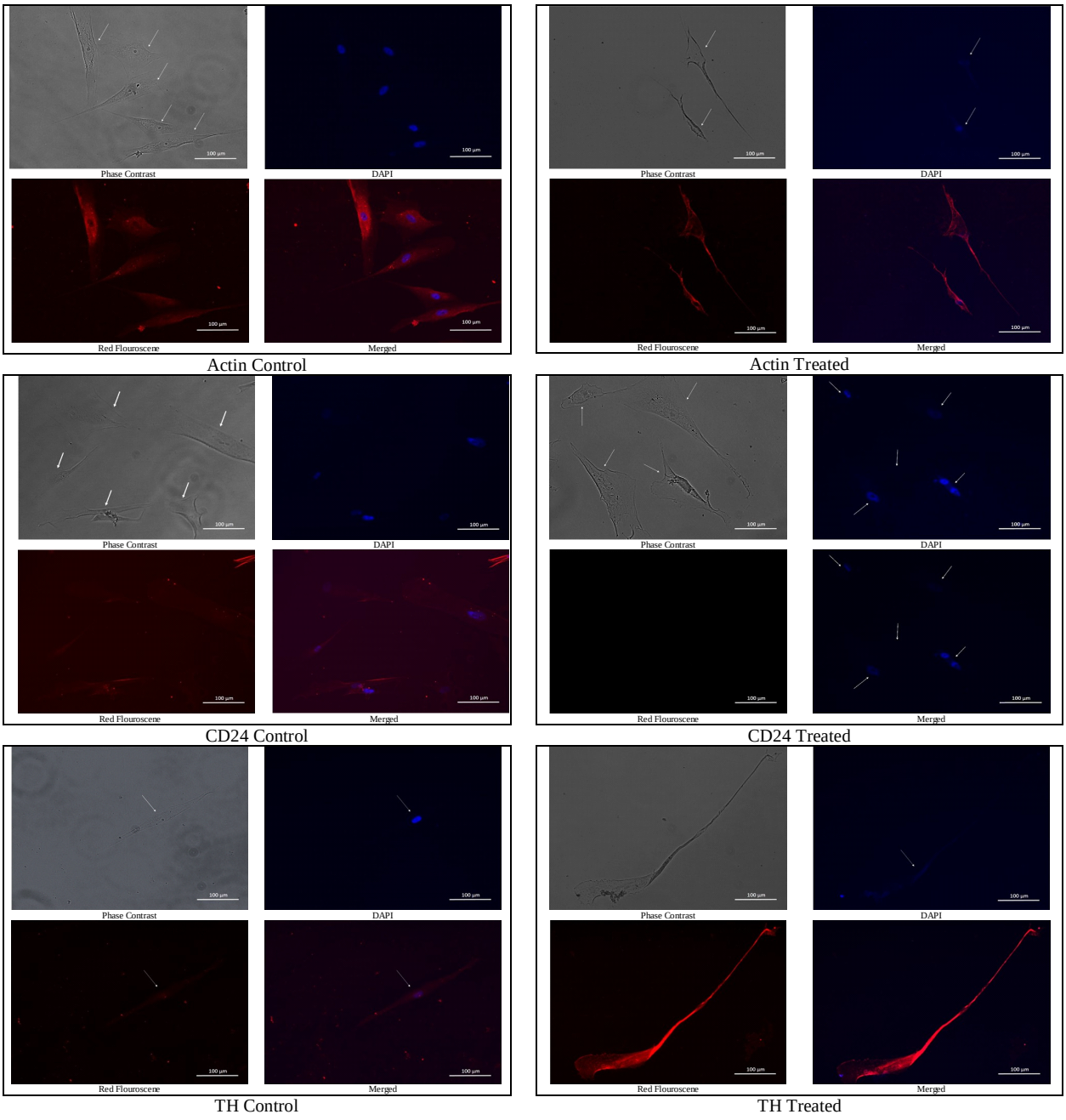

Figure-4: Immunocytochemical analysis of differentiated human umbilical cord derived mesenchymal stem cells

Actin showed positive in control and treated cells with neurogenic changes. The cells showed high expression of CD24 in the control and no expression in the treated cells. The Tyrosine protein showed low expression in the control whereas the Tyrosine protein showed highly expressed in the treated cells and exhibited dopaminergic neuron differentiation. Alexa fluor 546 anti-rabbit antibody was used for recognition. DAPI was used as stained in Nuclei. 100× magnification on Florid microscope.

DISCUSSION

The field of cell therapy and regenerative medicine holds great promise in restoring normal tissues structure and function. The main aim of stem cell based therapies is to target the chronic diseases and lifelong disabilities.[14] The best example of stem cell is MSCs, which have ability to differentiate into neuron-like cells and release different chemokines and growth factors. These growth factors and chemokines are necessary in revascularization, neurogenesis, immunomodulatory, anti-apoptotic and anti-inflammatory effects and have ability to engraft and migrate at sites of injury and inflammation.[15]

We have attempted to differentiate hUMSCs into neurons using Mevastatin, a commonly used antihyperlipidemic agent[16]. Mevastatin has potential to direct differentiate the neural progenitor cells into

dopaminergic neurons for the Parkinson's diseases.[12] These cells have been reported to have increased expression in proliferating hUMSCs previously.[17,18] In addition, we also checked for increased expression of the proteins Actin, Vimentin and CD24. These protein markers, especially Vimentin, were shown in multiple studies to be strongly positive.[17,19] They are also expressed in α-smooth muscle actin.[19]

At Mevastatin 1 µM concentration, characteristic properties of dopaminergic neurons in these cells, such as multipolar, unipolar and bipolar differentiation, star and diamond shaped cells, as well as elongated axon and/or dendrites were observed. These observations were validated in accordance with previous studies[20,21]. After confirmation of morphology, dopaminergic differentiation was further validated by demonstrating increased expression of NURR1, LMXIA and TH genes. These genes have been used demonstrating conversion of neuroprogenitor cells into dopaminergic neurons by using LY364947 and Mevastatin.[12]

Immunocytochemistry showed that the differentiated hUMSCs were positive for TH and Actin as well. However, a negative expression of CD24 was observed. These finding are in accordance with a similar experiment carried out on Wharton's Jelly derived mesenchymal stem cells differentiating into neuron-like cells using 1 mM concentration of Valproic acid.[22]

CONCLUSION

Mevastatin can differentiate hUMSCs into dopaminergic neuron like cells at 1 µM concentration. Using Mevastatin (when required) in those dyslipidemic patients who have increased susceptibility to Parkinson's disease is suggested. However, further research in this direction is recommended.

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Received: 8 Sep 2021 **Reviewed:** 10 Nov 2021 **Accepted:** 10 Nov 2021

Contribution of Authors:

HA: Original idea, sample collection and initial writing
SU: Sample processing and results, experimentation
STA: Proofreading and critical analysis
KH: Referencing, literature review and sample processing
SH: Data analysis, grammar and article writing and final detailing
KJJB: Inference, critical analysis and logical reasoning

Conflict of Interest: None declared
Funding received: None