Research Paper: Differentiation of Mesenchymal Stem Cells Derived From Human Adipose Tissue Into Cholinergic-like Cells: An in Vitro Study

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ABSTRACT

Introduction: Cholinergic-associated diseases currently constitute a significant cause of neurological and neurodegenerative disabilities. As the drugs are not efficient in improving the suffered tissues, stem cell treatment is considered an effective strategy for substituting the lost cells.

Methods: In the current study, we set out to investigate the differentiation properties of human Adipose-Derived Mesenchymal Stem Cells (AD-MSCs) into cholinergic-like cells by two morphogens of Retinoic Acid (RA) and Sonic Hedgehog (Shh) using a three-step in vitro procedure. The results were evaluated using real-time PCR, flow cytometry, and immunocytochemistry for two weeks.

Results: Our data showed that the cells could express cholinergic specific markers, including Islet-1, Acetylcholinesterase (AChE), SMI-32, and Nestin, at mRNA and protein levels. We could also quantitatively evaluate the expression of Islet-1, AChE, and Nestin at 14 days post-induction using flow cytometry.

Conclusion: Human AD-MSCs are potent cells to differentiate into cholinergic-like cells in the presence of RA and Shh through a three-step protocol. Thus, they could be a suitable cell candidate for the regeneration of cholinergic-associated diseases. However, more functional and electrophysiological analyses are needed in this regard.

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Highlights

- hAD-MSCs are potent cells to differentiate into cholinergic-like cells in the presence of RA and Shh through a three-step protocol.

- hAD-MSCs could express neural and cholinergic neuron-specific markers, including Nestin, SMI-32, Islet-1, and AChE in the presence of RA and Shh.

- Neural induction media containing RA for a total duration of Nine days and differentiated hAD-MSCs could express markers for immature and mature neurons.

Plain Language Summary

One of the diseases of Brains is cholinergic-associated diseases such as Alzheimer's disease. As the drugs are not efficient in improving the brain's damaged neurons, stem cell treatment is considered an effective strategy for recovering the lost cells. In the current study, we investigated the ability of human mesenchymal stem cells obtained from fatty tissues to differentiate into cholinergic-like cells by combining some agents. Our results showed that in the presence of these agents, mesenchymal stem cells obtained from fatty tissue could express cholinergic specific markers in the laboratory. It is concluded that human mesenchymal stem cells obtained from fatty tissue are capable cells that can differentiate into cholinergic-like cells. They are potent stem cells to be investigated to treat associated cholinergic diseases like Alzheimer's disease.

1. Introduction

Cholinergic-associated diseases are currently the major cause of neurological and neurodegenerative disabilities. Any cholinergic system dysfunction leads to various neurological diseases, such as Alzheimer Disease (AD) (Abdel-Salam, 2011). Currently, AD is the most prevalent neurodegenerative disorder and the sixth most common cause of death in the United States (Alzheimer's Association, 2015). The prevalence of the disease doubles every five years in people older than 65 years. This disease affects 30%–50% of all individuals by 85 years (Querfurth & LaFerla, 2010). It is the most common type of dementia with loss of cell nucleus cholinergic base (Alzheimer's Disease International et al., 2015).

So far, there is no cure for AD. Current treatments are divided into using medications, psychotherapy, and rehabilitation cares. The amount of acetylcholine, as the mediating chemical mediator in memory, is reduced in AD patients. Injection of acetylcholinesterase inhibitors has increased the cholinergic function and temporarily improves the disease (Borlongan, Sumaya & Moss, 2005; Castro & Martinez, 2006). Although various drugs are used to increase the amount of acetylcholine in the nerve terminals, they are not considered as definitive treatment because these drugs are associated with gastrointestinal complications (Schneider, 2000). Since the drugs are ineffective in treating and improving the suffered tissues, stem cell treatment is considered an effective strategy to recover the lost cells (Borlongan, 2012). Among various types of stem cells, Mesenchymal Stem Cells (MSCs) are of particular interest in regenerative medicine with regard to their self-renewal properties for a relatively long time (Chakari-Khiavi et al., 2019) and the ability to differentiate into multiple cell lineages, including osteoblasts (Darzi et al., 2012), chondrocytes (Khanmohammadi et al., 2012), adipocytes (Atmani, Chappard & Basle, 2003) and myocytes (Bana et al., 2017). They could also differentiate into various types of neural cell (Azedi et al., 2014; Azedi et al., 2017; Neuhuber et al., 2004; Yousefi, Sanooghi, Faghihi, Joghataei & Latifi, 2017; Zemelko et al., 2013), such as oligodendrocytes (Moayeri et al., 2017), motor neurons (Darvishi, Tiraihi, Mesbah-Namin, Delshad & Taheri, 2017), and even cholinergic cells (Marei et al., 2018).

The adipose tissue is distributed throughout the body and accounts for 15% to 20% of the body weight in men and 20% to 25% in women (Bulfer & Eugene Allen, 1979). Human adipose tissue is considered a medical waste product and an excellent source of mesenchymal stem cells. They can be easily obtained from fat tissue with minimal side effects. Human Adipose tissue Derived from Mesenchymal Stem Cells (hAD-MSCs) can differentiate into adipogenic (Gimble & Guilak, 2003),

osteogenic (El-Sayyad, Sobh, Khalifa & El-Sayyad, 2016), chondrogenic, and myogenic cells (Wickham, Erickson, Gimble, Vail & Guilak, 2003). They also have immunogenic (Kumar et al., 2012) and neuromuscular protecting properties (Tajiri et al., 2014). There are also some reports indicating that mesenchymal stem cells derived from adipose tissue can differentiate into neural cells (Choi, Cho, Seo, Yoon & Park, 2012; Jang, Cho, Cho, Park & Jeong, 2010; Marei et al., 2018; Qu, Liu, Song, Li & Ge, 2013; Radtke, Schmitz, Spies, Kocsis, & Vogt, 2009), such as motor neurons (Darvishi et al., 2017; Mohseni et al., 2019) and dopaminergic-like cells as well as cholinergic-like cells (Marei et al., 2018).

In this regard, our study aimed to investigate the differentiation properties of hAD-MSCs into cholinergic-like cells in the presence of Retinoic Acid (RA) and Sonic hedgehog (Shh) as two morphogens responsible for rostral-caudal specification (Wilson & Maden, 2005) of the neural tube, in vitro.

2. Methods

2.1. Isolation of human Adipose tissue Derived from Mesenchymal Stem Cells (hAD-MSCs)

The experiments were carried out according to the guidelines of the Ethics Committee of Iran University of Medical Sciences (Ethical code: IR.IUMS.REC1396.30694). After obtaining written consent from the healthy donors, fatty tissue was collected from a superficial layer of the abdomen during liposuction surgery of several individuals (age range: 25–46 years) at Motahari Hospital, Tehran City, Iran. The isolation of hAD-MSCs was performed according to Dubois et al. protocol (Dubois et al., 2008). Before setting out the isolation process, fatty tissue was warmed in a 37°C water bath. All isolation steps were then performed under the hood in sterilized conditions. The fat tissue was transferred to a tube containing 1% penicillin/streptomycin (Invitrogen) dissolved in warm Phosphate-Buffered Saline (PBS, Invitrogen). The sample was washed with PBS several times to eliminate blood and connective tissue. Then the fatty tissue sample was divided into small parts using sterilized scissors and was transferred to a tube containing 0.1% collagenase type I (Gibco, 17100-017, USA) and 1% Bovine Serum Albumin (BSA) (dissolved in warm PBS, Invitrogen) for digestion. Afterward, the sample was kept in a water bath for 30 min with shaking intervals to ensure total digestion. After digesting the tissue, the tube was centrifuged at 1200 rpm for 5 min at room temperature. After discharging the supernatant, the pellet was suspended in 1% BSA/PBS solution and centrifuged at 1200 rpm. To remove red blood cells, the Red Blood Cell (RBC) lysis buffer was used according to the manufacturer's protocol. After centrifugation, the cell pellet was suspended in a medium containing DMEM/Ham's F-12, 10% FBS, and 1% penicillin/streptomycin, then transferred to the tissue culture flasks (5000 cells/cm^2) and kept in a humidified chamber (temperature: 37°C, CO_2: 5 %, humidity: 98%). After forming colonies, the medium was exchanged with a fresh one every two days. At 70%-80% confluence, the cells were moved into three new flasks.

2.2. Characterization of hAD-MSCs

To characterize hAD-MSCs, the isolated cells at passage three were collected and incubated with fluorescent-conjugated monoclonal antibodies against CD90, CD73, CD45, CD44, CD29, CD105, and CD34 (all from BD Biosciences, USA) at 4°C for 40 min. The isotype-matched antibodies were used as control. The level of fluorescence greater than 95% of the one measured using the matched isotype control antibody was assumed as a positive expression. BD FACSCalibur flow cytometer (BD Biosciences, USA) was used to detect the expression of the antigens, and data analysis was performed by FlowJo software.

2.3. Induction of differentiation

Human AD-MSCs (1×10^5) were seeded in a 24-well plate containing the expansion medium and moved into a humidified chamber. Two days later, the medium was replaced with DMEM/F12, supplemented with 20% fetal bovine serum, 10 ng/mL basic fibroblast growth factor (FGF2, Sigma, USA), 2% B27 (Gibco, Germany), 250 mM isobutylmethylxanthine, and 100 mM β-mercaptoethanol for overnight. Afterward, the medium was changed with DMEM/F12, 0.2% B27 (Invitrogen, USA), 0.01 mM all-trans retinoic acid (RA; Sigma, USA), and 500 ng/mL Sonic hedgehog (Shh; R&D, USA). The medium was refreshed every 3 days. One week later, the cells were treated with DMEM/F12 and 0.2% B27 (Invitrogen, USA) for an additional 7 days. The medium was refreshed every 3 days.

2.4. Evaluation of differentiation

2.4.1. Immunophenotyping of differentiated hAD-MSCs

To quantify single properties of the differentiated cells, we evaluated the expression of Nestin, Islet-1, and Acetylcholinesterase (AChE) proteins by flow cytometry

at 14 days post-induction. To do that, the treated cells were incubated with fluorescence conjugated antibodies against human Nestin (Chemi-Con, USA), Islet-1 (cat# SC30200, Santa Cruz, USA), and AChE (cat# 3739, Abcam, USA) at 4°C for 30 min. Then, an FACSCalibur flow cytometer (BD BioSciences) was used to detect expressions of these proteins. Positive expression was defined as the level of fluorescence greater than 95% of the one measured using the corresponding isotype-matched control antibodies. The results were analyzed using FlowJo software.

2.4.2. Cytofluorimetric analyses of the expression of neural markers in differentiated hAD-MSCs

After 14 days of differentiation, the cells were fixed in acetone at -20°C for 2 min. The fixed cells were blocked with 5% sheep serum for 10 min at room temperature and then incubated for 90 min at room temperature with human Nestin (1:300; Chemi-Con, USA), Islet-1 (cat# SC30200, Santa Cruz, USA), and AChE (cat# 3739, Abcam, USA). Subsequently, the cells were washed three times with 0.1% PBS/BSA and incubated with Fluorescein Isothiocyanate (FITC)-labeled sheep anti-rabbit IgG or sheep anti-mouse IgG at room temperature for 45 min in the dark. The nuclei were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride at 1 µg/mL for 5 min. After washing, the coverslips were mounted in 70% PBS-glycerol and examined under a fluorescence microscope (Olympus, Tokyo, Japan).

2.4.3. Gene expression

Real-time PCR was carried out to determine the expression of the candid genes at 7 and 14 days of induction. Briefly, total cellular RNA was extracted using TRIzol reagent (Invitrogen, Germany). After digestion of DNA, the purity of RNA was quantified spectrometrically. Complementary DNA (cDNA) was synthesized using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, Canada). Real-time Polymerase Chain Reaction (PCR) reactions were carried out using the 7500 real-time PCR system (Applied Biosystems, USA). In each PCR reaction, ×1 SYBR Green PCR Master Mix (Applied Biosystems, USA) was mixed with 12 ng of cDNA and the related primers (Table 1) in a total volume of 20 mL. The expression of Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH) as an internal control was used to normalize the expression levels of the target genes in the treated cell groups compared with the same genes in hAD-MSCs at passage three.

2.5. Statistical analysis

The obtained data were presented as the Mean±Standard Error of Mean (SEM). The differences between study groups (n=3 in each group) were statistically assessed by nonparametric Mann-Whitney test in SPSS version 16. P values less than 0.05 were considered significant.

3. Results

3.1. Isolation and characterization of hAD-MSCs

The isolated cells could express CD105, CD29, CD90, CD73, and CD44, while they were negative for CD45, CD31, CD34, and HLADR markers (Figure 1).

3.2. Evaluation of differentiation

3.2.1. Immunostaining

After induction of hAD-MSCs in a three-step protocol using RA and Shh, the cells could express neural and cholinergic antigens, including Nestin, AChE, and Islet-1, at 14 days post-induction (Figure 2).

3.2.2. Cytofluorimetric analysis

Flow cytometry was used to evaluate the number of cells expressed cholinergic markers quantitatively. According to our data, mean±SD cell numbers of 17.78±3.86, 36.025±6.76, and 52.165±6.51 could express Nestin, AChE, and Islet-1, respectively (Figure 3).

3.3. Gene expression

Gene expression analyses of hAD-MSCs treated with RA and Shh showed significant upregulation of candid genes, including Nestin as neural progenitor cell marker and Islet-1 and AChE as two cholinergic markers at 7 days post-induction when compared with non-treated MSCs at passage three as the control ($P<0.05$). The expression of the candid genes decreased one week later at 14 days post-induction, remarkably (Figure 4).

4. Discussion

Loss of cholinergic neurons in AD has declined the quality of life in many patients worldwide. Several studies indicated the efficiency of stem cell therapy in the regeneration of cholinergic neurons. Nowadays, human AD-MSCs are considered a therapeutic agent in neurodegenerative disorders and one of the interesting cellular sources for stem cell therapies. These cells have valuable properties such as self-renewal, immunomodulatory

Table 1. Sequences of the primers

Name of Genes	Primer Sequence (5'-3')	NCBI Accession Number
Nestin	(F) AAAGTTCCAGCTGGCTGTGG (R) CCAGCTTGGGGTCCTGAAA	NM_006617.2
Islet-1	(F) ATATCAGGTTGTACGGGATCAAATG (R) R CACGCATCACGAAGTCGTTC	NM_002202.2
AChE	(F) GCAGGAGAAGACAGCCAACT (R) TGCAAACCTCAGCTGGTCAT	NM_020549.4

NEUROSCIENCE

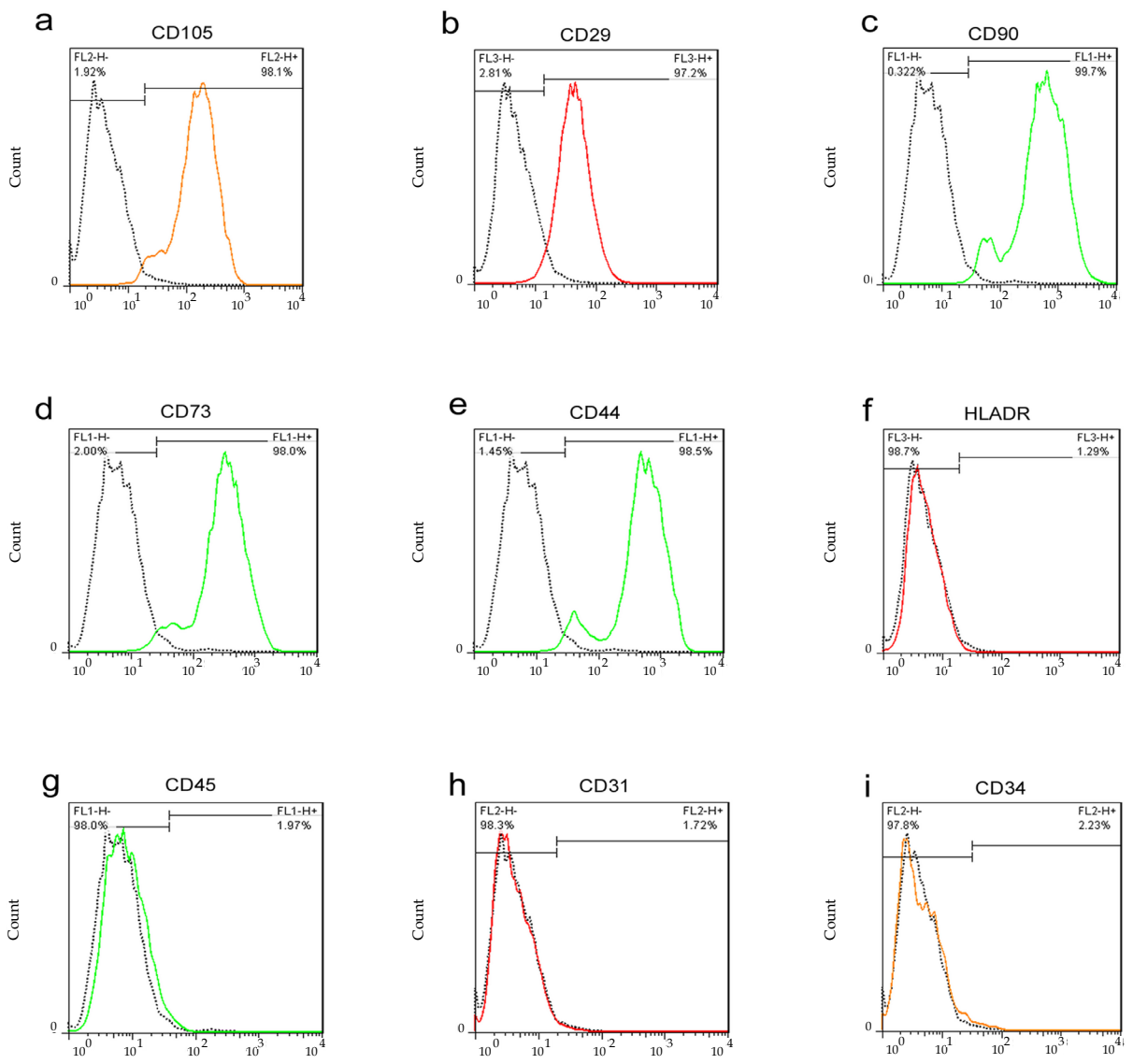

Figure 1. Isolation and characterization of the isolated cells

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For the characterization of the cells, representative illustration of the candid CDs has been tested in cultured cells at passage three. The respective isotype control is shown as a gray line.

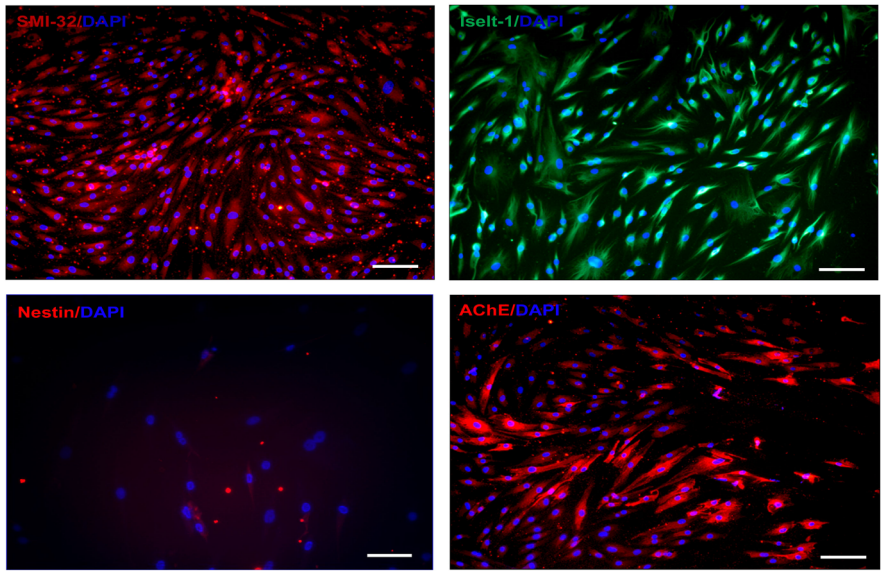

Figure 2. Evaluation of cholinergic differentiation by cytofluorimetric analysis

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The expression of SIM-32, Nestin, AChE, and Islet-1 has been detected by immunocytochemistry at day 14 post-induction. Nuclei are counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (blue). Scale bar 50 µm (×20).

characteristics, and differentiation potential into many lineages, particularly neural cells. To our knowledge, there are not enough reports about cholinergic differentiation of AD-MSCs using in AD treatment. Furthermore, improved protocols for MSCs induction will facilitate and ensure the reproducibility and standard production of MSCs for therapeutic applications in neurodegenerative diseases.

In our research, we demonstrated that isolated hAD-MSCs expressed typical levels of MSCs markers like CD105, CD29, CD90, CD73, and CD44. On the other hand, the absence of expression of CD45, CD31, CD34, and HLADR markers distinguished AD-MSCs from hematopoietic and endothelial cell types (Amini et al., 2016).

To obtain cholinergic neurons from AD-MSCs, we implicated cholinergic differentiation by using RA and Shh. It is well established that RA has an essential role in neural differentiation (Janesick, Wu & Blumberg, 2015). RA has been implicated in two aspects of spinal cord dorsoventral patterning. Firstly, in the induction of a subset of ventral interneurons (Pierani, Brenner-Morton, Chiang & Jessell, 1999) and secondly, in the specification of mo-

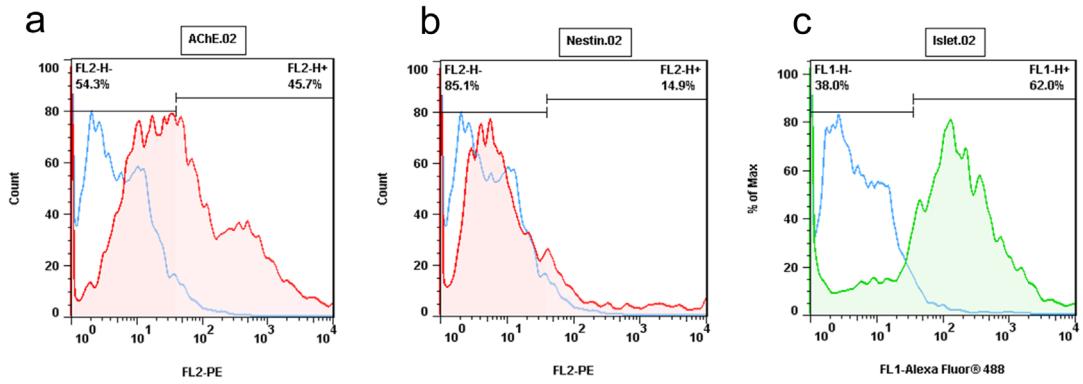

Figure 3. Evaluation of cholinergic differentiation by cytofluorimetric analysis

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The expression of Nestin, AChE, and Islet-1 has been detected by Flow cytometry at day 14 post-induction. The respective isotype control is shown as a blue line.

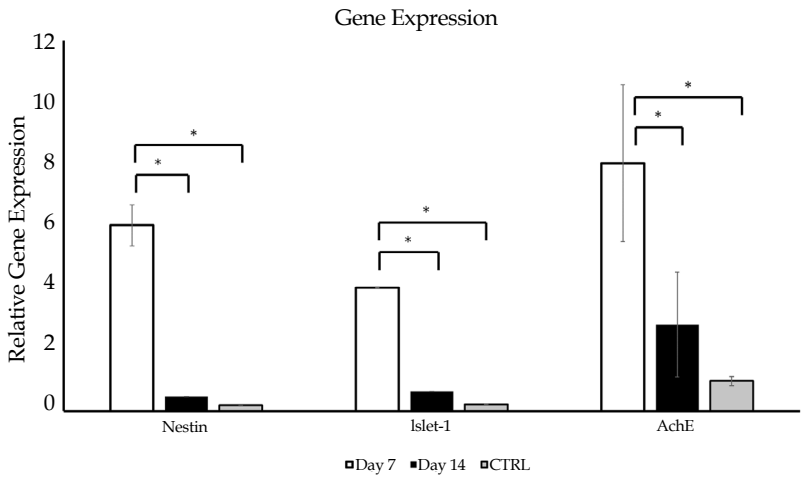

Figure 4. Gene expression

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The relative expression of Nestin, AChE, and Islet-1 was analyzed at 7 and 14 days post-induction. Human AD-MSCs at passage three were assumed as the control cell. Remarkable upregulation of the candid genes was observed on day 7 when the data were compared with the Control (P<0.05). The level of expression decreased one week later, on day 14.

tor neuron subtypes (Sockanathan & Jessel, 1998). Also, Shh plays a critical role in the patterning of the vertebrate embryonic nervous system, including the brain and the spinal cord, during development. Moreover, Shh acts in a graded fashion to pattern the dorsal-ventral axis of the vertebrate spinal cord (Chiang et al., 1996).

Based on our results, hAD-MSCs could express neural and cholinergic neuron-specific markers, including Nestin, SMI-32, Islet-1, and AChE at the protein level in the presence of RA and Shh. SIM-32 is a nonphosphorylated neurofilament (NP-NF) marker and can serve as a relatively reliable marker of motor neurons (Gotow & Tanaka, 1994). It is well established that loss of NP-NF such as SIM-32 is associated with AD (Thangavel, Sahu, Van Hoesen & Zaheer, 2009). Therefore, increasing this marker in differentiated AD-MSCs can enhance their potential capacities in AD treatment.

Besides, we showed that differentiated AD-MSCs could express AChE, Nestin, and Islet-1 at the protein level. Marei et al. (2018) also showed that using neural induction media containing RA for a total duration of 9 days and differentiated MSCs could express markers for immature and mature neurons (Marei et al., 2018). AChE, the enzyme responsible for acetylcholine degradation to acetate and choline, has long been considered a marker of cholinergic neurons (Zoli, 2000). When we evaluated the gene expression of neural and cholinergic neuron-specific markers in differentiated AD-MSCs, we observed that AChE, Nestin, and Islet-1 increased highly after 7 days; however, these values decreased one week later at 14 days. Increasing of islet-1 was reported by previous studies after neural induction by Shh and RA (Darvishi et al., 2017; Faghihi et al., 2016).

5. Conclusion

Overall, our results suggested that hAD-MSCs have a great capacity to differentiate into cholinergic-like cells. Human AD-MSCs have some advantages over other sources of MSCs, including simple extraction procedures, convenient cultivation, high abundance, high rate of proliferation, neuroprotective, and immunomodulatory effects (Frese, Dijkman & Hoerstrup, 2016). Indeed, developing protocols for MSCs induction to cholinergic neuron needs more comprehensive studies. In this regard, more animal studies and applying functional techniques such as patch-clamp for better generalization are suggested.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the Iran University of Medical Sciences (Code: IR.IUMS.REC.1396.30694).

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Authors' contributions

All authors equally contributed to preparing this article.

Conflict of interest

The authors declared no conflict of interest.

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