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Upregulation of Neurotrophic Factors and Myelin Basic Protein in Schwann-like Cells by T3 Hormone Following Transdifferentiation of Human Adipose-derived Stem Cells

Giti Zarinfard, Maryam Aliakbari, Vajihe Asgari, Shahnaz Razavi*

Department of Anatomical Sciences, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.

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ABSTRACT

Peripheral nerve regeneration is a complicated phenomenon. Thyroid hormones are known as critical regulators in the nervous system development. The Schwann cells have the regenerative potency in the peripheral nervous system. In this study, the human adipose-derived stem cells were assessed *in vitro*, for transdifferentiation potency into Shwann-like cells (SLCs) as a candidate source for clinical cell therapy, under the treatment of triiodothyronine (T3) hormone, and compared with the untreated cells. The cell viability rate, myelination and neurotrophic factors expression of SLCs were evaluated two weeks post- induction by MTT assay, immunocytochemistry and real-time RT-PCR techniques, respectively. The obtained results revealed a significant decrease in SLCs viability, compared to the adipose-derived stem cells ($P < 0.001$). Immunocytochemistry technique was applied to detect SLCs markers, such as S100β, GFAP and myelin basic proteins (MBP) in the presence and absence of T3 treatment. The results indicated that administering T3 can significantly increase the differentiation and myelination potency of SLCs ($P < 0.01$). The findings of real-time RT-PCR technique indicated that the expression of Schwann cells markers, MBP, brain-derived neurotrophic factor and glial cell-derived neurotrophic factor were upregulated significantly with T3 hormone administration in comparison with the untreated cells ($P < 0.05$). The SLCs were able to express the neurotrophic factors and myelination related genes in the presence of T3 hormone. Furthermore, T3 administration improved myelination potency of adipose-derived stem cells, *in vitro*. Further *in vivo* experiments are necessary to confirm the advantages of using a combination of autologous SLCs and T3 hormone for peripheral nerve injury recovery.

Keywords: Adipose-derived stem cells, myelin, neurotrophic factors, Schwann cells, thyroid hormone

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***Corresponding Author:** Shahnaz Razavi
Address: .*Department of Anatomical Sciences, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.*
E-mail: razavi@med.mui.ac.ir

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Introduction

Nerve injury in human is usually accompanied by long-term deficiencies. Applying cell-based therapy to repair nerve injury is one of the treatment strategies. Also, a combination of a variety of cellular and molecular components could be applied for nerve regeneration.

Schwann cells (SCs) constitute the main contributing non-neuronal cells, in the peripheral nervous system. The significance of SCs in cell therapies for nerve injury repair is subject to their myelinating ability, neurotrophic factors (NTFs) synthesis, expression of cell adhesion molecules and extracellular matrix (ECM) (1). Consequently, SCs are believed to exert a regenerative influence during nerve injury and neurodegenerative disease (2, 3). However, the slow expansion rate and the difficulties in harvesting have limited the application of SCs for cell transplantation (4).

Mesenchymal stem cells are the promising candidate stem cells in regenerative medicine. Adipose-derived stem cells (ADSCs) are a type of adult stem cells, which can be easily obtained through low-invasive protocols. ADSCs are abundant source of adult stem cells, have lower immunogenicity, show long-term survival, and are able to differentiate into different cell lineages (5). The microenvironment of ADSCs is very important, as various factors are involved in differentiation potency. Adding some agents like growth factors, antioxidants, cytokines and extracellular components, are required for ADSCs induction into differentiated ADSCs (dADSCs) (6-8). ADSCs can be differentiated into Shwann–like cells (SLCs) with similar SCs properties (9, 10). After peripheral nerve injury, several factors are required for nerve regeneration, including ECM, neurotrophic factors, and hormones (11).

ECM is very important for SCs function, and consists of specialized macromolecules and proteins, including fibronectin and laminin. These glycoproteins are critical for SCs proliferation, differentiation, survival, and SC morphology regulation (12, 13).

Neurotrophic factors are proteins with high contribution in enhancing neurogenesis during peripheral nerves development and repair (14). Some of the most important neurotrophic factors produced by SCs consist of the brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), glial cell-derived neurotrophic factor (GDNF), and nerve growth factor (NGF). ADSCs can produce neurotrophic factors and induce peripheral nerve regeneration without being differentiated into SLCs (15, 16). The dhADSCs transplant improved survival and myelin formation in comparison with undifferentiated hADSCs transplant. Moreover, hADSCs induced into SLCs, were able to produce neurotrophic factors more than that of the undifferentiated hADSCs (17). In the presence of laminin, the hADSCs produce more neurotrophic factors during differentiation into SLCs, which promote myelination *in vitro* (18).

Thyroid hormones are essential for development and maturation of the brain and normal brain function throughout life, as well as for peripheral nerve development (11). Also, THs influence the expression of myelin proteins: proteolipid protein (PLP), myelin basic protein (MBP) and myelin- associated glycoprotein (MAG) (19). Moreover, THs were shown to promote *in vitro* differentiation and maturation of oligodendrocyte precursors, induced from rat neural stem cells (20).

HADSCs are multipotent adult stem cells, capable of being induced in a variety of differentiated cells such as adipocytes, chondrocytes, osteocytes and neural cells upon induction by THs (21, 22). On the other hand, IL12P80 (homodimer of interleukin-12 subunit p40) could replace or have a synergistic effect with

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ciliary neurotrophic factor (CNTF) and triiodothyronine (T3) hormone to induce neural stem cells into myelinating SCs, which in turn promote nerve regeneration (23).

The purpose of this study was to evaluate the *in vitro* efficacy of T3hormone on hADSCs differentiation into SLCs, together with myelination potency and neurotrophic factors expression, in SLCs cells.

Materials and Methods

Experimental design

In order to assess the effects of T3 on transdifferentiation of hADSCs into SLCs, human adipose tissue was obtained from subcutaneous abdominal fat. After that, hADSCs (3-4 passages) were affected by neural induction medium containing T3, and induction medium without T3 considered as a control group. To evaluate the effect of T3, on SLCs differentiation of hADSCs, qRT-PCR analysis of neural marker genes (glial fibrillary acidic protein (*GFAP*), S100 calcium binding protein B (*S-100β*), *MBP*, *BDNF*, *GDNF*, *NGF*, *CNTF*) was carried out for treated and untreated induced cells and also, GFAP, MBP and *S-100β* were evaluated at 2 week post-induction of hADSCs with immunocytochemistry assay (Figure 1).

Human ADSCs isolation and culture

In this experimental study, all protocols were approved by the Ethics Committee Supervising Procedures at Isfahan University of Medical Sciences, Isfahan, Iran. The human adipose tissue was obtained from subcutaneous abdominal fat of four female donors, with an age range of 25–47 years after receiving informed consent.

First, the subject tissue samples were rinsed by phosphate-buffered saline (PBS), containing 2% penicillin/streptomycin (P/S), followed by removal of connective tissue and blood vessels from fat tissue. Afterwards, the chemical digestion was performed in 0.01 % collagenase type I (Sigma-Aldrich, St.Louis, MO, USA) solution for 30 min at 37 °C in 5 % CO_2. Next, the enzymatic digestion was neutralized by adding DMEM: F12 (1:1), supplemented with 10 % fetal bovine serum (FBS), and centrifuged for 10 min at 1700 rpm. The cell pellet was re-suspended by medium and then plated in T25 plastic flasks, containing DMEM: F12 medium 10 % FBS and 1% P/S. Then, the flasks were incubated in a humidified tissue culture incubator, with 5 % CO_2 at 37 °C.

After 24 h, the non-adherent cells were discarded, while the attached cells were expanded. The ADSCs were harvested, using 0.25% trypsin/EDTA (Gibco, BRL, Paisley, UK) for 3 min at 37 °C to obtain a single cell suspension, and replated through the serial passage to generate a homogenous cell population. In this experiment, cells were used after 3-4 passages.

Human ADSCs differentiated into Schwann-like cells

The hADSCs stemness was confirmed by flow cytometry and the *in vitro* differentiation of hADSCs into SLCs was carried out according to the previous study (8). This differentiation was performed in two steps, including induction of hADSCs to the neurosphere where hADSCs were plated in plastic tissue culture plates in a DMEM: F12 (1:1) medium, supplemented with 20 ng/mL human epidermal growth factor (hEGF) (Sigma-Aldrich, St.Louis, MO, USA) , 20 ng/mL human basic fibroblast growth factor (bFGF) (Sigma-Aldrich, St.Louis, MO, USA), and 2% B27. The medium was refreshed every 2-3 days. After seven days of plating, the neurospheres were dissociated with pipetting via 0.25% trypsin/EDTA.

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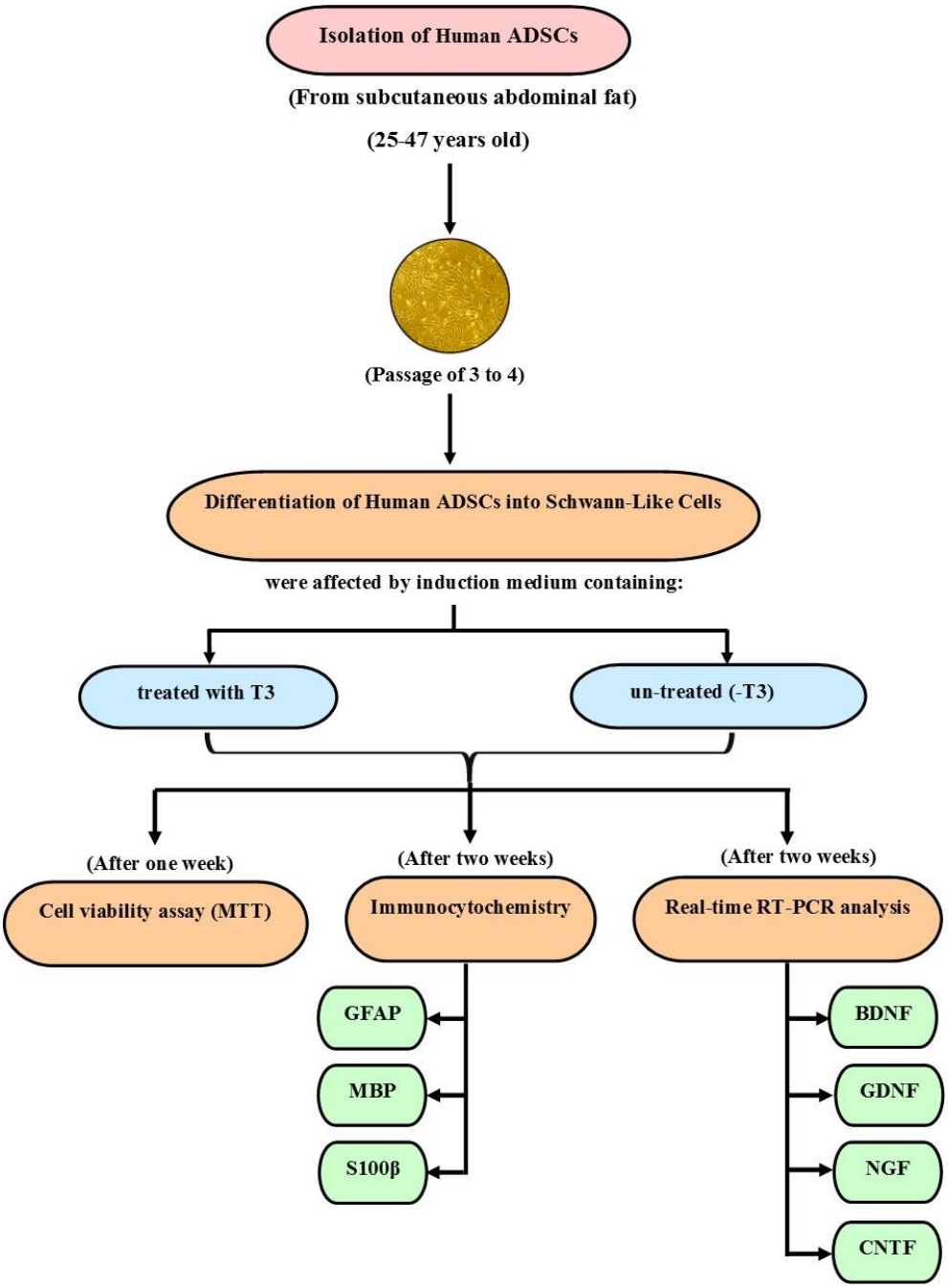


Fig. 1. Schematic illustration of the procedure used to assess the effects of T3 on transdifferentiation of hADSCs into Schwann-like Cells.

The single cells were replated in the presence of terminal differentiation DMEM: F12 /10 % FBS medium supplemented with 5 µm forskolin (Sigma-Aldrich, St.Louis, MO, USA), 5 ng/mL platelet-derived growth factor-AA (PDGF) (Sigma-Aldrich, St.Louis, MO, USA), 10 ng/mL bFGF (Sigma-Aldrich, St.Louis, MO, USA), and 200 ng/mL recombinant human heregulin-beta (Sigma-Aldrich, St.Louis, MO, USA) for seven

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days. In this step, T3 with 50 nM concentration was added to cell culture flask in treated group (+T3), but not to the control group (-T3).

MTT assay

The cell viability of SLCs was determined using 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT assay), in the presence or absence of T3 hormone, post induction. After cell counting, 4×10^3 cells were plated in each well of a 24-well plate. After 7 days, the differentiated cells were washed with PBS, then 5 mg/mL MTT solution was added to the DMEM: F12 medium, at 1:10 dilution, and incubated with 5% CO_2 at 37 °C for 4 h. After discarding this medium, the formazan crystals were dissolved by adding 400 µL DMSO solution, until purple color was observed in each well through pipetting. The absorbance was detected by a micro plate reader (Hiperion MRP 4+, Germany) at 540 nm wavelength. The absorbance values were related to the number of living cells.

Immunocytochemistry analysis

To determine the effect of T3 hormone on the differentiation potency of SLCs, the expression of some markers, GFAP and S100β SCs markers were evaluated. Also, myelinating ability was assessed through assessing MBP marker by immunocytochemistry. The differentiated cells were fixed in 4% paraformaldehyde for 20 min at room temperature (RT), permeabilized with 0.2 % Triton x-100 in PBS for 30 min at RT, followed by incubation with primary antibodies, overnight at RT [anti- S-100β (1:500, Abcam, UK), (anti- GFAP, 1:300, Abcam, UK) and (anti- MBP, 1:500, Abcam, UK)].

Afterward, the samples were rinsed and incubated with conjugated secondary antibody, rabbit anti-mouse FITC (1:500, Abcam, UK), and rabbit anti-mouse PE (1:1000, Abcam, UK) at RT for 60 min. Then, DAPI solution (1:1000, Sigma) was added for labeling the nuclei. Experiments were performed in triplicate, fluorescence images were captured by fluorescence microscope (Olympus BX51, Japan). The images were analyzed, using the Image J software, the number of immunopositive cells were counted in several non-overlap fields, and a minimum of 200 cells per slide were determined.

Real-time reverse transcription polymerase chain reaction (Real time RT-PCR)

The gene expression was evaluated, using the real-time reverse transcription polymerase chain reaction (RT-PCR) technique (24). The total RNA was extracted from SLCs, using the High Pure RNA isolation kit (Roche, Germany), according to the manufacturer's protocol. The RNA was assessed by spectrophotometric analysis using a Nanodrop (Nano spec cube, Nanolytic, Germany). The extracted RNA from each sample was reversely transcribed with DNase I (Roche, Germany), to eliminate genomic contamination. Isolated RNA was converted into complementary DNA (cDNA), using the Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) and oligo dT primer. The primers for all assessed genes were designed using Allele ID 7.6 (Primer Biosoft). All primers' sequences (forward-reverse) and real-time RT-PCR program were presented in (Table 1). After DNA amplification, real-time RT-PCR was run via gene specific primers, Maxima SYBR Green/ROX qPCR Master Mix 2X (Thermo Scientific, USA) and the Steponeplus™ real-time RT-PCR detection system (Applied Biosystems, USA). To verify the specificity of reaction, a melting curve analysis was used to determine the melting temperature. The genes were normalized against the housekeeping gene, *GAPDH*. These experiments were run in triplicates. The $2^{-\Delta\Delta CT}$ method was adopted to determine the level of relative expression for each gene.

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Table 1. Primer sequences (forward, reverse) used in RT-PCR analysis.

Gene Symbol	Description	Function*	Sequence (5`to 3`)	Accession No.
GFAP	Glial Fibrillary Acidic Protein	Intermediate filament (IF) protein, structural molecule activity and structural constituent of cytoskeleton, as a marker to distinguish astrocytes from other glial cells.	CCGACAGCAGGTCCATGTG GTTGCTGGACGCCATTGC	NM_001131019
S-100β	S100 Calcium Binding Protein B	Regulation of a number of cellular processes such as cell cycle progression and differentiation.	GGAGACGGCGAATGTGACTT ACTCGTGGCAGGCAGTAGTAA	NM_006272
MBP	Myelin Basic Protein	Major constituent of the myelin sheath of oligodendrocytes and Schwann cells in the nervous system.	GGCCCCGTGGATGGA GAGGCGCGAAAGGAGATG	NM_001025090
BDNF	Brain Derived Neurotrophic Factor	Promotes neuronal survival in the adult brain. This gene may play a role in the regulation of the stress response and in the biology of mood disorders.	AGCTCCGGGTTGGTATACTGG CCTGGTGGAACTTCTTTGCG	NM_170734
GDNF	Glial Cell Derived Neurotrophic Factor	To promote the survival and differentiation of dopaminergic neurons in culture, and was able to prevent apoptosis of motor neurons induced by axotomy.	TCAAATATGCCAGAGGATTAT CCTG GCCATTTGTTTATCTGGTGAC CTT	NM_199231
NGF	Nerve Growth Factor	Nerve growth stimulating activity and regulation of growth and the differentiation of sympathetic and certain sensory neurons.	CATGCTGGACCCAAGCTCA GACATTACGCTATGCACCTCA GTG	NM_002506
CNTF	Ciliary Neuronotrophic Factor	Survival factor for neurons and oligodendrocytes and may be relevant in reducing tissue destruction during inflammatory attacks.	CCTGACTGCTCTTACGGAATC CTAT CCATCCGCAGAGTCCAG	NM_000614
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase	Catalyzes an important energy-yielding step in carbohydrate metabolism.	GAAATCCCATCACCATCTTCC AGG GAGCCCCAGC CTTCTCCATG	NM_002046

*: Gene functions were adopted from GeneCards®: The Human Gene Database

Statistical analysis

The statistical analysis of data was performed using SPSS software version 20. All the data in different groups were compared through ANOVA, followed by Tukey, s post hoc test. The data were presented as the mean value ± standard error (mean ± SEM) and the levels of the statistical significance were expressed as P-values (*$P \leq 0.05$, **$P \leq 0.01$, and ***$P \leq 0.001$).

Results

Cell culture and change in morphology of isolated hADSCs

By seeding ADSCs into cell culture flask, many cells were attached to the bottom. After 2-3 passages, the isolated ADSCs were found as a homogenous population with typical flattened fibroblast-like morphology (Figure 2A).

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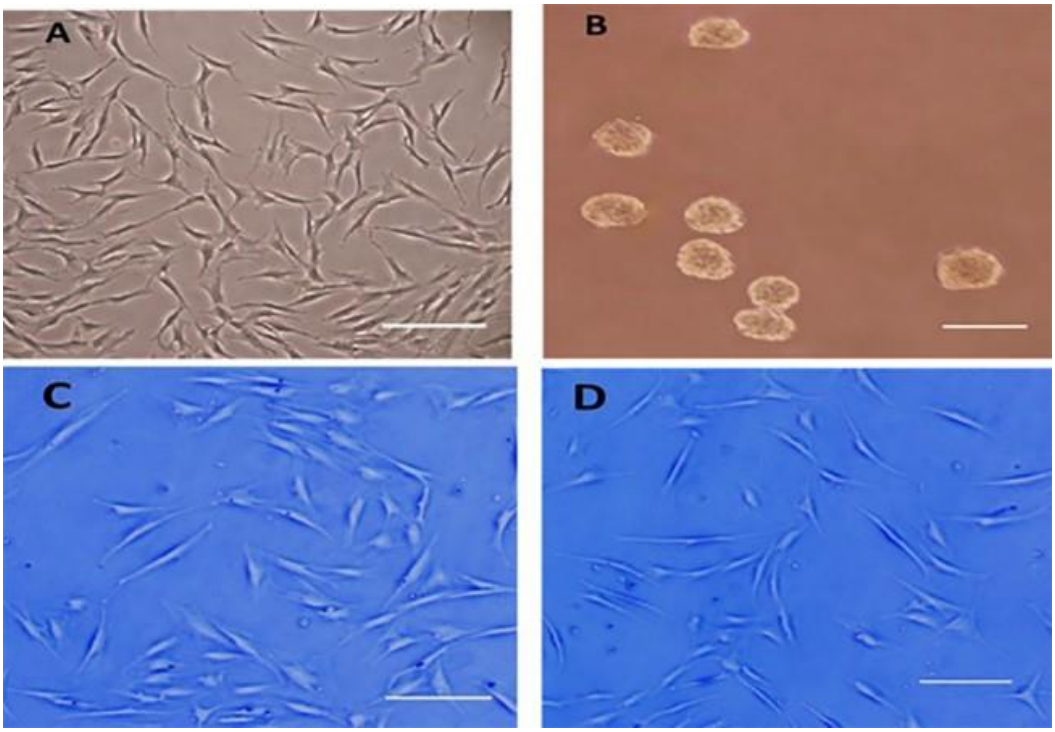


Fig. 2. Morphological characteristics and differentiation of human ADSCs by inverted phase contrast microscopy. A: h ADSCs adhering to tissue culture plastic flask showing fibroblast - like morphology; B: Neurospheres formation in tissue culture dish, seven days post-induction (step one). The final differentiation into an elongated spindle or triangular shape typical of SCs (dADSCs), two weeks post-induction (step two); C: Cell differentiation upon treatment with T3 hormone; D: Control without T3 hormone treatment. Scale bars in A= 200 µm; B = 150 µm; C and D = 100 µm.

Morphological changes following SLCs differentiation

The gradual changes in cell morphology in all stages were observed, using phase contrast microscopy. HADSCs were induced to neurosphere structures by re-plating in pre-induction medium, where after seven days clumps of floating cells were observed, as shown in (Figure 2B).

The neurospheres were detached from the substrate, dissociated, and then the final differentiation was performed, with or without T3 hormone. The differentiated cells with a spindle-like or triangular shape morphology were shown in Figure 2C and D.

Evaluation of the Cell viability

The effect of T3 hormone in promoting cell viability of SC-like cells was assessed through MTT assay. The mean absorbance of stem cells, T3- treated and untreated groups were 0.509 ± 0.048, 0.265 ± 0.040 and 0.210 ± 0.014, respectively. Our results showed that the mean of cell viability of the stem cell group was significantly more than the differentiated cell groups (***p≤ 0.000), while no significant difference was found in the mean of cell viability between T3- treated SC-like cells, compared to the untreated group.

Immunofluorescence analysis

HADSCs were induced into SLCs, and to determine the effect of T3 on cell induction, immunocytochemical staining was performed using Schwann cell markers (S100β, GFAP) and myelin basic protein (MBP), two weeks after induction. The expression of these markers was illustrated in two groups (Figure 3 and Figure 4).

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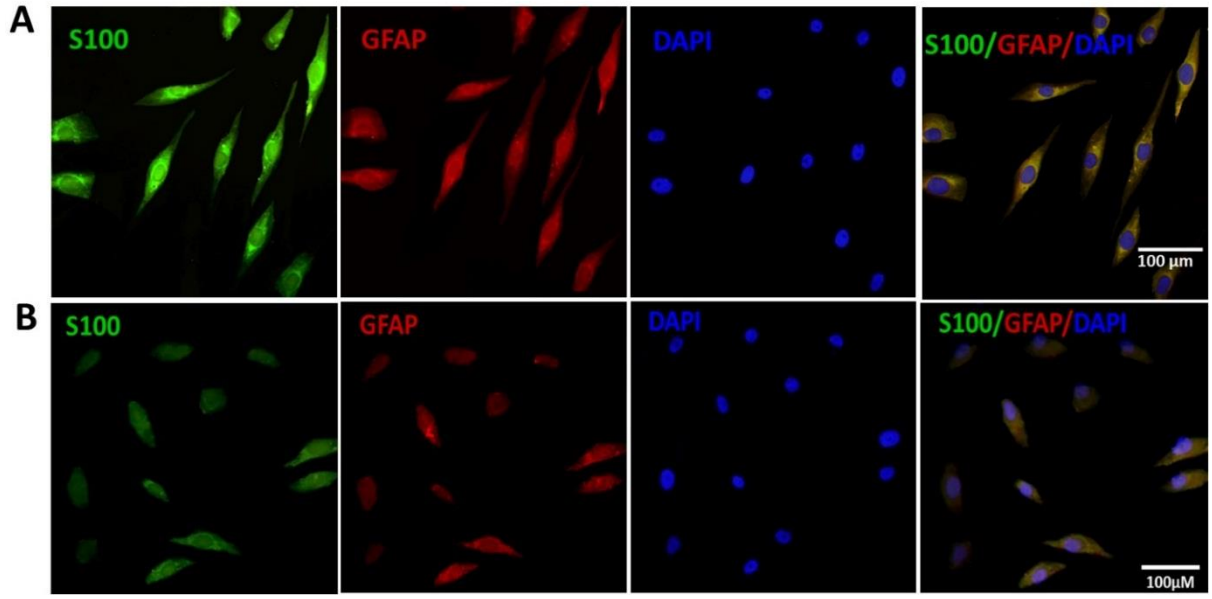


Fig. 3. Immunocytochemical staining of SLCs, post-induction and after treatment of hADSCs. A: In the presence of T3 hormone; B: In the absence of T3 hormone. Specific SCs markers were used. S100β (green), GFAP (red) and (S100β/GFAP co-markers, yellow to brown). The yellow to brown color was an overlap of green and red, showing positively stained cells for each marker, in each experiment the nuclei were counterstained with DAPI (blue). Scale bars= 100 µm.

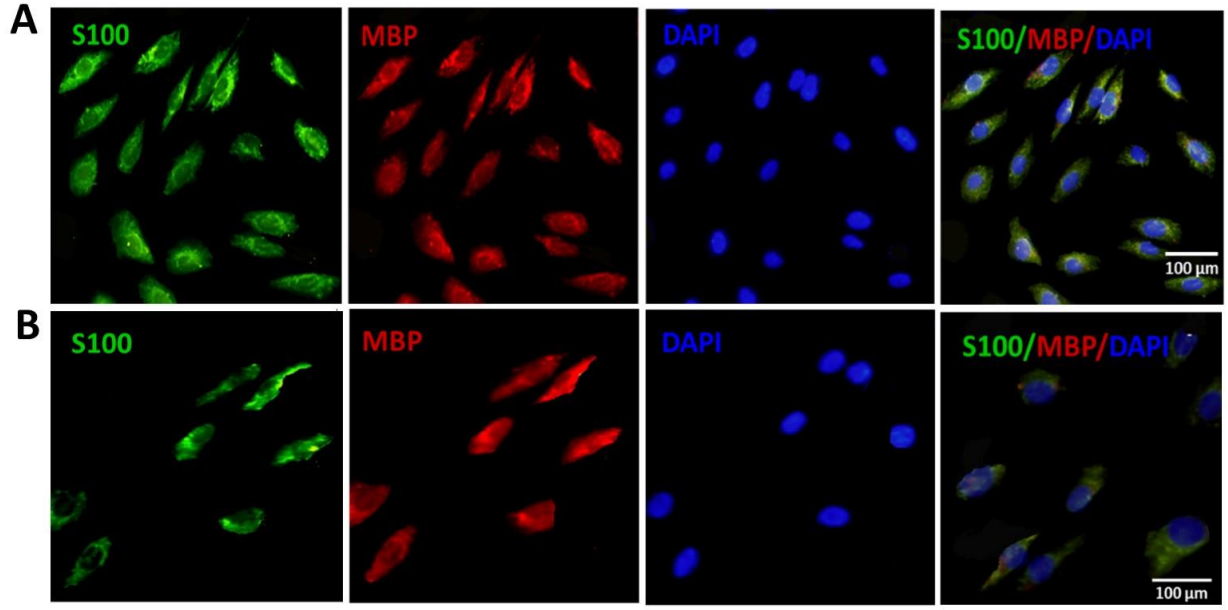


Fig. 4. Immunocytochemical staining of SLCs, post-induction and after treatment of hADSCs. A: In the presence of T3 hormone; B: In the absence of T3 hormone. Specific SCs markers were used. S100β (green), myelinating marker MBP (red) and S100β/MBP co-markers, yellow to brown). The yellow to brown color was an overlap of green and red, showing positively stained cells for each marker, in each experiment the nuclei were counterstained with DAPI (blue). Scale bars= 100 µm.

Immunofluorescence analysis revealed that the mean percentage of co-markers, S100β/GFAP positive cells increased significantly in the T3- treated group (75.50 ± 3.24%) in comparison with the untreated group

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(61.46 ± 4.55 %) (*P < 0.05). The mean percentage of differentiated cells expression of co-markers, S-100β/MBP in the T3- treated group was higher (50.87 ± 2.16 %) in comparison with the untreated group (47.75 ± 5.35 %); however, this increase was not significant (Figure 5 A).

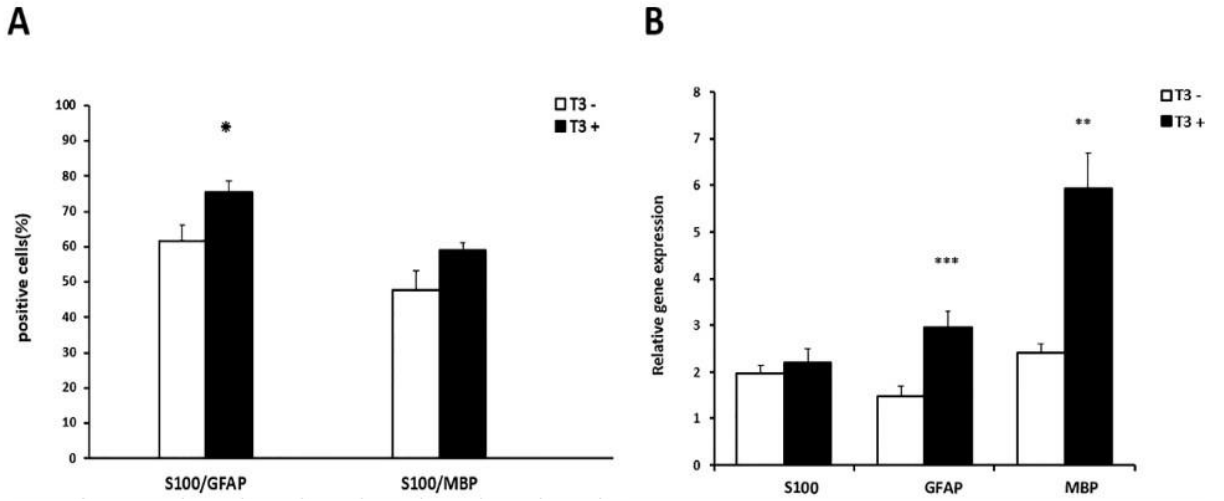


Fig. 5. Comparative analysis of the mean percentages of immunopositive cells for SCs and myelinating markers. A: There was a significant increase in SCs markers *S100β/ GFAP*, in the presence of T3 hormone, compared to the untreated group (mean ± SEM, *P < 0.05); B: Real- time RT-PCR results were used to reveal the changes in gene expression for SCs markers (*S100β*, *GFAP*) and *MBP*. The expression of *GFAP* was significantly increased in the treated cells (mean ± SEM, ***P < 0.001). Similarly, T3-treated cells showed a significant increase in *MBP* expression, compared to the untreated cells (mean ± SEM, **P < 0.01) in SLCs derived from human ADSCs in T3- treated and untreated conditions.

Real-time RT-PCR analysis

To confirm the level of gene expression in SLCs, real-time RT-PCR was applied two weeks post-induction to determine the effects of T3 hormone on cell differentiation, myelinating potency and the expression of neurotrophic factors, such as *BDNF*, *NGF*, *CNTF*, and *GDNF* in SLCs. The genes were normalized with *GAPDH*, followed by assessing the levels of gene expression of some markers in T3-treated and untreated groups.

Although, the *S100β* gene expression was up regulated in T3- treated group (2.21± 0.30), compared to the untreated group (1.72 ± 0.176), this up regulation was not significant. A significant up regulation for *GFAP* was observed in T3-treated group (2.96 ± 0.34), compared to the untreated group (1.47 ± 0.191) (P < 0.001). The myelination potential of SLCs was determined, where *MBP* expression in the T3- treated group (5.93± 1.44) was significantly higher than its counterpart (2.04 ± 0.23) (P < 0.01) (Figure 5 B).

The *BDNF* and *GDNF* expressions were up regulated with T3 hormone treatment; the expression of *BDNF* in the T3- treated group was significantly up regulated (4.34 ± 1.30), in comparison with the untreated group (1.61 ± 0.178) (P < 0.05). Here, the results indicate that the *GDNF* expression was significantly up regulated in the T3- treated group (5.53 ± 0.57), in comparison with its counterpart (1.45 ± 0.28) (P < 0. 001).

Our findings indicate that the *NGF* expression was down regulated in the T3- treated group (0.49 ± 0.063), and in untreated group (0.59 ± 0.075), compared to the undifferentiated cells. In contrast, the *CNTF*

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expression was up regulated, in the treated group (3.25 ± 1.56), compared to the untreated group (2.57 ± .097); however, the mean difference of *CNTF* between the two groups was not significant (Figure 6).

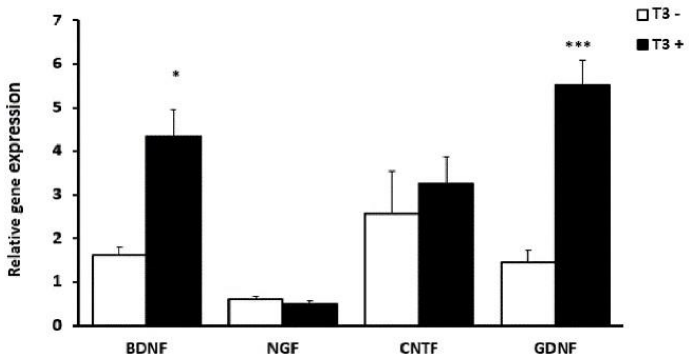


Fig. 6. Gene expression analysis of neurotrophic factors. Real-time RT-PCR analysis was used to reveal the changes in gene expression for neurotrophic factors such as *BDNF*, *NGF*, *CNTF* and *GDNF* markers in SLCs derived from human ADSCs in T3 -treated and untreated conditions. The expression of *BDNF* and *GDNF* was significantly upregulated in SLCs in the treated cells, compared to the untreated control (mean ± SEM, *P < 0.05, ***P < 0.001).

Discussion

Several factors are necessary for an appropriate neuronal function, such as SCs, extracellular matrix, neurotrophic factors and hormones. It has been reported that the T3 hormone can immediately effect on both the axotomized neurons and SCs, in transected rat sciatic nerve, which may display an important role for T3 hormone in nerve regeneration (25). in addition, it has been suggested that the treatment of impaired peripheral nerves with biodegradable guides, in the presence of T3 leads to an increase in the number of axons and myelin sheath thickness and also a refined regeneration of the sciatic nerve (26). Thyroid hormones exert their function through nuclear thyroid receptors (TRs). Vertebrates have two TR subtypes, the TRα and TRβ, which are homologous and ligand dependent. A wide range of mammalian TRs exists, but only TRα1, TRβ1 and TRβ2 isoform can be transcribed actively (27).

In the nervous system, expression pattern of TRs depends on the region and type of neural cell as well as the developmental stage (28). TRs in neurons and glial cells of central nervous system are predominantly expressed during embryonic and adult life, whereas, in glial cells of peripheral nervous system like SCs, expression of TRs is limited to periods of development and repair. The nuclei of SCs do not express TRs in young and adult rats (29). While TRβ-1 is distributed all over the gray matter of the central nervous system, TRα-1 is the predominant isoform in SCs; the expression of which is established during the sciatic nerve development, TRα-1 isoform is inactivated during myelination process and can no longer be detected (30). It has been reported that after transection of the sciatic nerve, SCs are able to express TRα-1 isoform, binding of T3 to TRα-1 may regulate the proliferation of SCs and the expression of molecules, inducing myelin-related proteins, following nerve injuries. The TR subtype switches from TRα to TRβ in the nervous system; cell proliferation is stimulated by TRα while, the migration and differentiation are induced by TRβ (29).

Moreover, in sciatic nerve cell cultures, SCs remarkably express mainly TRα-1. The TRα presents predominantly in multipotent human ADSCs, where TRα-1 regulates cell cycle- associated processes, while, TRβ is involved in hADSCs differentiation processes (31).

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In the present study, we have evaluated the cell survival, using MTT assay. Based on our findings, cell viability in SLCs decreases, compared to the hADSCs. ADSCs differentiated into SLCs in a time-dependent manner, many of the ADSCs underwent apoptosis and failed to differentiate into SLCs. The cell viability in differentiated groups decreased gradually with induction time, compared to the undifferentiated group (ADSCs) (32). Previous reports could be assigned to the developmental stages and an exchange from TRα expression to TRβ expression stage, in other words, a switch between proliferation and differentiation (31). Moreover, it has been reported that T3 treatment during hADSCs neural induction, suggests that the cell viability rate of differentiated cells, in the treated group was significantly more than the untreated group (33). In addition, we observed higher cell viability in T3-treated SLCs, in comparison with the untreated group, but the difference was not statically significant.

Immunocytochemical method was performed for the detection of S100 and GFAP proteins as markers of SCs and MBP for myelinating potency. GFAP is an important intermediate filament protein in glial cells, astrocytes in the central nervous system, and SCs in peripheral nervous system. The GFAP is expressed in immature SCs but later on it will be suppressed, when SCs produces myelin (34). Therefore, in differentiated myelin-forming SCs, GFAP has low expression, but this ability is maintained in SCs. Another report suggests that at axotomized condition, SCs return into non-myelin-forming SCs with a potential to express GFAP (35). According to our results, administration of T3 hormone, significantly promoted co-expression of S100 and GFAP, in comparison with the untreated group. However, the immunocytochemistry analysis showed that the MBP expression did not significantly increase between the two groups. Therefore, the myelinating potency of SLCs increased at a non-significant rate in T3- treated group, compared to the untreated groups.

In addition, real-time RT-PCR analysis was performed to detect SCs markers and *MBP* at the mRNA level; the results of our experiment indicated a significant upregulation in the expression of *GFAP* and *MBP*, following SLCs induction in the presence of T3 hormone in comparison with the untreated group. That could be an indicator of the role of T3 hormone in promoting SLCs differentiation. Moreover, a previous study reported that myelin-related genes such as *MBP* were direct T3-responsive genes (36).

Furthermore, the neurotrophic expressions were evaluated. Neurotrophins constitute the main factors that are able to support neuronal survival and other cells in the nervous system. Neurotrophins such as BDNF, NT-3, NT-4 and GDNF are important for cell survival and neurite outgrowth, in particular during development, but they are down-regulated in differentiated neural cells (37). The expression of *BDNF* and *NT-3* are thyroid hormone- dependent. It has been reported that in hypothyroidism, the level of *BDNF* expression decreases (38). During the injuries of the adult nervous system, *BDNF* levels decrease in central nervous system and peripheral nervous system, while the use of thyroid hormones after injury becomes critical for *BDNF* up regulation (39).

Our real-time RT-PCR results demonstrated a significant up regulation of *BDNF* and *GDNF* expression, by evaluating the neurotrophic factors following treatment with T3 hormone, compared to its counterpart, while the up regulation of *CNTF* was not statistically significant between the two groups. In addition, the *NGF* expression was down regulated in the presence and absence of T3 hormone.

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It has been shown that the expression of *BDNF* is low until day 3 after lesion, and then gradually reaches its maximum rate in 3–4 weeks. On the contrary, the *NGF* expression increases to a maximum level rapidly, 24 h after injury and it reaches its maximum rate up to day 3, and then follows a degradation pattern (32). In this context, different culture conditions and discrepancies in media collection, in different time intervals may affect these results.

The main objective of this experiment was to evaluate the effects of T3 hormone on SLCs differentiation, using a human autologous source of ADSCs. Based on our findings, T3 administration improves myelination potency of dADSCs, i*n vitro*. We also assessed the expression of neurotrophic factors during this differentiation process. Based on our results, the expression of some neurotrophic factors increased in SLCs during treatment with T3. However, further *in vivo* experiments are necessary to confirm the advantages of using a combination of autologous SLCs and T3 hormone transplantation, in accelerating the recovery of peripheral nerve injuries and neurodegenerative disorders.

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Conflicts of Interest

We declare no conflict of interest.

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