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## Dimethyl fumarate up-regulates expression of major neurotrophic factors in the epidermal neural crest stem cells

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### ABSTRACT

There is an agreement that combining treatments can lead to substantial improvement, therefore the present study assessed the effects of different concentrations of dimethyl fumarate (DMF) on viability of epidermal neural crest stem cells (EPI-NCSCs). In addition, this investigation was designed to evaluate the effects of DMF on relative expression of major trophic factors mainly the ones with neurotrophic effects, expressed in EPI-NCSCs in order to enhance their therapeutic potential. To determine the appropriate concentration of DMF for EPI-NCSCs treatment, the MTT assay was employed and based on the obtained data, EPI-NCSCs treated with 10 $\mu$ M DMF for 6, 24, 72 or 168 h. In each time point, quantitative RT-PCR technique was used to evaluate NGF, NT-3, BDNF, GDNF and VEGF transcripts. The acquired data showed that 10 $\mu$ M DMF significantly increased the mRNA expression of NGF, NT-3 and BDNF, 72 h following treatment; however, DMF inhibitory effect on GDNF mRNA expression was observed in various time points. No significant changes were detected for VEGF transcript. Our findings revealed that expression of major neurotrophic factors were up-regulated by dimethyl fumarate treatment. Therefore, combining EPI-NCSCs with DMF treatment might be a valuable strategy to improve their therapeutic functions *in vivo*.

### 1. Introduction

Over the past few decades, stem cell therapy has witnessed a surge in investigating and treating numerous diseases affecting various organs. Stem cells have proven to be an attractive option for treating neurological diseases and the list of stem cells therapeutic applications in clinical practice is rapidly growing. However, owing to limited restorative potential of cellular therapy and complication of neurological disorders, it is well established that combining treatments can lead to significant improvement. In this regard, therapeutic approaches that combine drugs with cell transplantation, attenuating the growth inhibitory pathways and/or activating growth promoting programs have been explored (Park et al., 2010). On the other hand, stem cells therapy for neurological diseases have been utilized for decades (Sakthiswary and Raymond, 2012; Shroff, 2018), but the existing therapies are merely able to manage the condition and attenuate its progression and fail to treat them completely.

Dimethyl fumarate (DMF) is an ester of fumaric acid, frequently

prescribed for the treatment of multiple sclerosis (MS) relapsing forms. Currently, DMF is approved as a first-line of monotherapy for MS and it has shown to significantly reduce the development of active brain MRI lesions and relapse rate following DMF treatment (Fox et al., 2012; Gold et al., 2012). In addition, DMF reduces cerebral edema formation and protects the integrity of blood–brain barrier (Kunze et al., 2015), modulates the immune/inflammatory response, promotes neuronal survival and rescues neurological function upon ischemic stroke (Lin-Holderer et al., 2016; Lin et al., 2016; Safari et al., 2017).

During the embryonic development, neural crest stem cells migrate to variety of tissues and these stem cells persist into adulthood. Several lines of evidence reported that adult neural crest-derived stem cells such as dental pulp stem cells are suitable choice for regenerative medicine in craniomaxillofacial damages due to their regenerative potential (La Noce et al., 2014; Romeo et al., 2018). Epidermal neural crest stem cells (EPI-NCSCs) are another multipotent neural crest stem cells, present in the bulge of adult hair follicle. These cells are neural precursor cells that retain the neurologic differentiation potential of

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**Table 1**  
Primer sequences (5′–3′) used in quantitative polymerase chain reaction (qPCR).

Gene	Sequence	Amplicon (bp)
<i>Ngf</i>	F- CCCAATAAAGGCTTGCCAAGGAC R- GAACAACATGGACATTAGCTATGC	78
<i>Nt-3</i>	F- GACACAGAACTACTACGGCAACAG R- ACTCTCCTCGGTGACTCTTATGC	184
<i>Bdnf</i>	F- CGATTAGGTGGCTTCATAGGAGAC R- CAGAACAGAACAGAACAGAACAGG	182
<i>Gdnf</i>	F- GCTGACCAGTGACTCCAATATGC R- CCTCTGGCCTTTCCTCTG	192
<i>Vegf</i>	F- ACTTGAGTTGGGAGGAGGATGTC R- GGATGGGTTTGTCTGTTTCTGG	183
<i>Actin, Beta</i>	F-TCTATCTGGCCTCACTGTC R-AACGCAGCTCAGTAACAGTCC	122

their neural crest origin (Krejčí and Grim, 2010). EPI-NCSCs was initially introduced by Sieber-Blum et al. (2004) as a promising donor cells for diverse cell therapy paradigms due to its advantages, such as no graft rejection, easy accessibility with minimal invasive procedure, adequate obtaining source, multipotency, and high degree of inherent plasticity as well as not having ethical issues (Li et al., 2017). Furthermore, EPI-NCSC express variety of trophic factors, including neurotrophins, extracellular proteases and angiogenic factors that have the capability of supporting cell survival and neo-vascularization (Sieber-Blum, 2010).

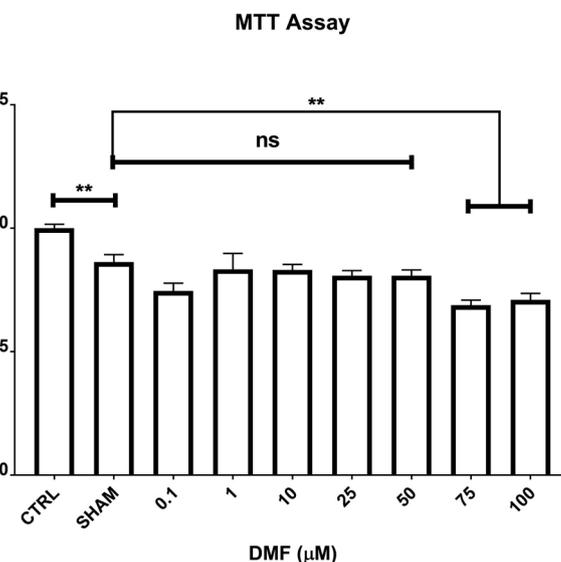
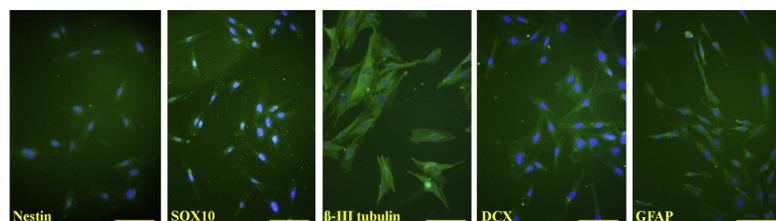
Hence, this type of stem cells might be a good alternative for treating neurological diseases instead of mesenchymal or neural stem cells since their effectiveness was reported in spinal cord injury (Hu et al., 2010; Pandamooz et al., 2018), peripheral nerve injuries (Li et al., 2017) and Alzheimer's disease (Esmailzade et al., 2012).

Accordingly, combination of EPI-NCSCs therapy with DMF treatment might be a valuable strategy for reducing the devastating conditions of neurological disorders. Nevertheless, it is necessary to examine the effects of DMF on EPI-NCSCs *in vitro*, ahead of employing their combinational therapy *in vivo*. Although there is a great interest to use DMF in treatment of neurological diseases, there are few studies that have investigated the impacts of DMF on trophic factors expressed in stem cells. Hence, in the present study, first the effects of different doses of DMF on viability of EPI-NCSCs was assessed to define proper and safe concentration for further *in vitro* evaluations. Then, the study was designed to evaluate the impacts of DMF on relative expression of major trophic factors in EPI-NCSCs, mainly the ones that have great influence on the central nervous system development and regeneration in order to enhance their therapeutic function.

## 2. Materials and methods

### 2.1. Isolation of EPI-NCSCs

The bulges of hair follicles were micro-dissected from the adult rat whiskers pad and isolated bulges were placed on collagen coated 4-well cell culture plate. The explants were fed with minimum essential medium  $\alpha$  ( $\alpha$ -MEM, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 5% day-11 chick embryo extract and 1%



**Fig. 2.** Viability of EPI-NCSCs following 72h incubation with various doses of dimethyl fumarate (DMF). Sham treated group incubated with 0.1% DMSO. \*\* $P < 0.01$ ; ns: not significant.

penicillin/streptomycin (Gibco, USA), and were incubated in a humidified atmosphere at 37 °C with 5% CO<sub>2</sub>. The details of this procedure were described elsewhere (Pandamooz et al., 2013; Sieber-Blum and Grim, 2004). After EPI-NCSCs migration, cells were detached using 0.25% trypsin/EDTA (Gibco, USA).

### 2.2. Verification of isolated EPI-NCSCs

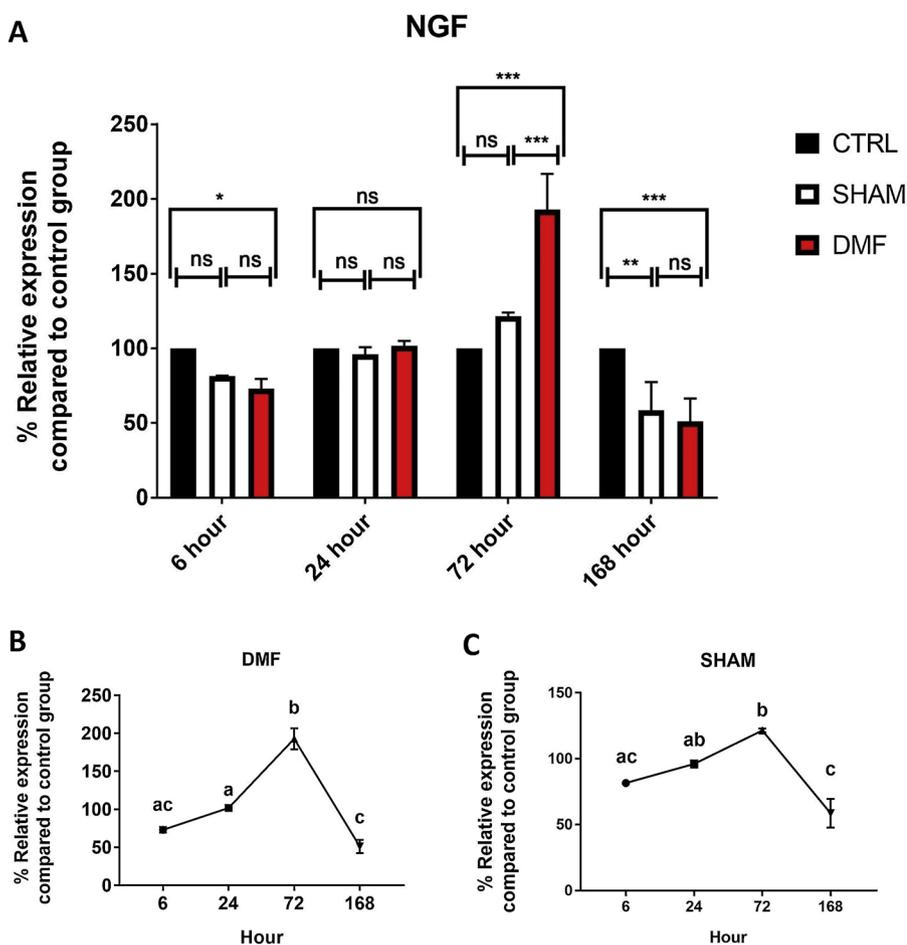
Verification of expanded EPI-NCSCs was performed using immunofluorescent staining against nestin (a marker of neural crest stem cells and undifferentiated cell), SOX10 (a marker of neural crest cells), doublecortin (DCX),  $\beta$ -III tubulin (markers of immature neurons) and GFAP (glial marker). In brief, cultured EPI-NCSCs were fixed in 4% paraformaldehyde and washed with TPBS (0.05% Tween-20 in PBS). After blocking with 1% bovine serum albumin in 0.2% triton X-100, cells were incubated with primary antibodies overnight in cold condition (4 °C).

The following primary antibodies were used: rabbit anti-nestin (Abcam, #ab93157, USA), rabbit anti-SOX10 (Abcam, #ab155279, USA), rabbit anti-  $\beta$ -III tubulin (Abcam, #ab18207, USA), rabbit anti-doublecortin (Abcam, #ab77450, USA) and rabbit anti- GFAP (Abcam, #ab7260, USA). After three washes with TPBS, cells were incubated with FITC conjugated secondary antibody (Sigma, #F1262, USA) and counterstained with DAPI (Sigma, #D9564, USA).

### 2.3. Cell viability assay

To determine the appropriate concentration of DMF for EPI-NCSC treatment, the MTT assay was employed based on the previously introduced doses of DMF, which is used to treat other types of stem cells (Ghods et al., 2013; Wang et al., 2015). Here, twenty-four hours before drug treatment, the stem cells were seeded in 96-well plate in triplicate.

**Fig. 1.** Immunostaining against nestin, SOX10,  $\beta$ -III tubulin, doublecortin (DCX) and glial fibrillary acidic protein (GFAP) to verify migrated cells. Cell nuclei counterstained with DAPI. Scale bar: 100  $\mu$ m.



**Fig. 3.** Relative expression of NGF mRNA levels in EPI-NCSCs at various time points following treatment. A) Statistical comparison between groups in each time point. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; ns: not significant. B and C): Statistical comparison in each treatment between various time points. Values with different superscripts are significantly different ( $P < 0.05$ ).

Next day, plates medium was replaced with culture medium containing 0.1, 1, 10, 25, 50, 75 and 100  $\mu\text{M}$  DMF (prepared in  $\alpha$ -MEM containing 0.1% DMSO, 10% FBS and 1% penicillin/streptomycin) and cells were incubated for 6, 24, 72 and 168 h with this drug at 37 °C. At the end of these time points, cells medium were removed and 0.5 mg/ml MTT (Sigma, USA) prepared in  $\alpha$ -MEM was added to each well. Following 4 h incubation, the MTT solution was discarded and acidic isopropanol added to dissolve blue formazan crystals. Finally, the developed color was measured at 570 nm using microplate (ELISA) reader (BioTek, USA).

#### 2.4. DMF treatment

The day before DMF treatment, EPI-NCSCs were seeded at density of  $10^6$ ,  $5 \times 10^5$ ,  $8 \times 10^4$  and  $2 \times 10^4$  cells/well in two different 6-well plates for 6, 24, 72 and 168 h treatments, respectively. Based on acquired data from the MTT assay and previous examinations, EPI-NCSCs were treated with 10  $\mu\text{M}$  DMF for 6, 24, 72 or 167 h. In addition, each time point of the treatment had its own corresponding control and sham groups, which were incubated with  $\alpha$ -MEM containing 10% FBS plus 1% penicillin/streptomycin or  $\alpha$ -MEM containing 0.1% DMSO, 10% FBS and 1% penicillin/streptomycin, in the same culture condition.

#### 2.5. RNA extraction, cDNA synthesis and qRT-PCR

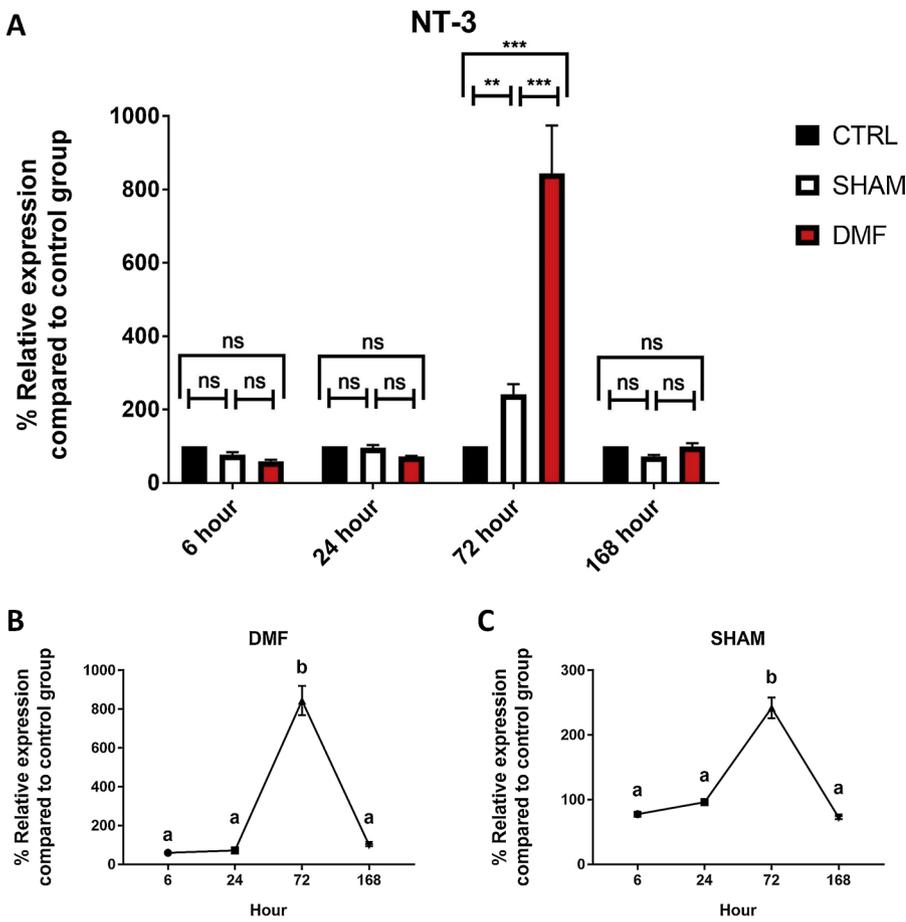
At the end of each time points, the total RNA was extracted from EPI-NCSCs culture using Ytzol (Yekta Tajhiz Azma, #YT9063, Iran). Following treatment of total RNAs with DNase I (Thermo Scientific, USA), the DNase-treated RNAs were employed to synthesize cDNA using cDNA Synthesis Kit (Yekta Tajhiz Azma, #YT4500, Iran). All

procedures were performed as described by the suppliers.

For qRT-PCR, triplicate reactions for measurement of nerve growth factor (NGF), neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF) and vascular endothelial growth factor (VEGF) mRNA levels were carried out on the cDNA samples using gene-specific primers presented in Table 1. Reactions consisted of RealQ Plus Master Mix Green (Ampliqon, Denmark), first strand cDNA template and appropriate qRT-PCR primer set. Thermo-cycling conditions were: 95 °C for 15 min followed by 35 cycles of 95 °C for 15 s and 60 °C for 60 s performed on an ABI StepOne Real-Time PCR system (Applied Biosystems, USA). Melting curve analysis showed a single amplification peak for each reaction. The Ct value for each target was normalized to the copy number of the beta-actin transcript for each sample. Furthermore, a single band of the expected size was observed for amplified products (5  $\mu\text{l}$ ) that were subjected to electrophoresis on a 1% agarose gel stained with ethidium bromide. Fold changes in expression were calculated using the arithmetic formula  $2^{-\Delta\Delta\text{CT}}$  (Livak and Schmittgen, 2001).

#### 2.6. Statistical analysis

Target genes relative expression data were subjected to the normality test. Comparisons between groups (control, sham and DMF treated group) and time points (6, 24, 72 and 168 h) were made by two-way analysis of variance plus the Tukey posthoc test using GraphPad Prism (Version 7.03, GraphPad Software, Inc., San Diego, CA). The mean  $\pm$  SEM are reported in the text and  $P < 0.05$  was considered to be statistically significant.



**Fig. 4.** Relative expression of NT-3 mRNA levels in EPI-NCSCs at various time points following treatment. A) Statistical comparison between groups in each time point.  $**P < 0.01$ ;  $***P < 0.001$ ; ns: not significant. B and C): Statistical comparison in each treatment between various time points. Values with different superscripts are significantly different ( $P < 0.05$ ).

### 3. Results

#### 3.1. The characteristics of migrated EPI-NCSCs

In the present study, two-three days following explantation, the migrated cells were detected around the bulges. Seven days after migration, immunostaining against specific markers of EPI-NCSC revealed the expression of nestin, SOX10, DCX,  $\beta$ -III tubulin and GFAP, which verified the type of migrated cells as an EPI-NCSCs (Fig. 1).

#### 3.2. Viability of EPI-NCSCs treated with DMF

In the current investigation, the MTT assay was employed to confirm the safety of DMF and its proper concentration for EPI-NCSCs treatment. The acquired data revealed the viability of EPI-NCSCs following treatment with  $10\mu\text{M}$  DMF to be very close to sham group. Accordingly, DMF at the concentration of  $10\mu\text{M}$  was selected for further evaluation of its impacts on the expression level of various trophic factors' transcripts. Fig. 2 shows the results of MMT assay following 72 h EPI-NCSCs treatment with various doses of DMF. The MTT results after 168 h of treatment was almost the same; however, in 6 and 24 h post treatments no significant changes were observed (data not shown).

#### 3.3. DMF up-regulated the transcripts of major neurotrophic factors -NGF, NT-3 and BDNF- in the EPI-NCSCs

Quantitative RT-PCR analysis revealed,  $10\mu\text{M}$  DMF significantly increased the mRNA expression of NGF, 72 h following treatment; however, this concentration reduced NGF mRNA levels at 6 and 168 h after incubation. Also, the same pattern was observed in the sham group though with lesser impact (Fig. 3). Furthermore, we found that

72 h DMF treatment elevated the level of NT-3 mRNA in the EPI-NCSCs by around 800%. It is worth noting that NT-3 expression was unaffected in the other time points, and sham group also showed likewise pattern, but with lesser impact (Fig. 4). Furthermore, the BDNF mRNA level was another target that its expression slightly but significantly induced by DMF, at 24 and 72 h after treatment. However,  $10\mu\text{M}$  DMF declined the BDNF transcript at the time point of 6 h and was ineffective at the time point of 168 h (Fig. 5).

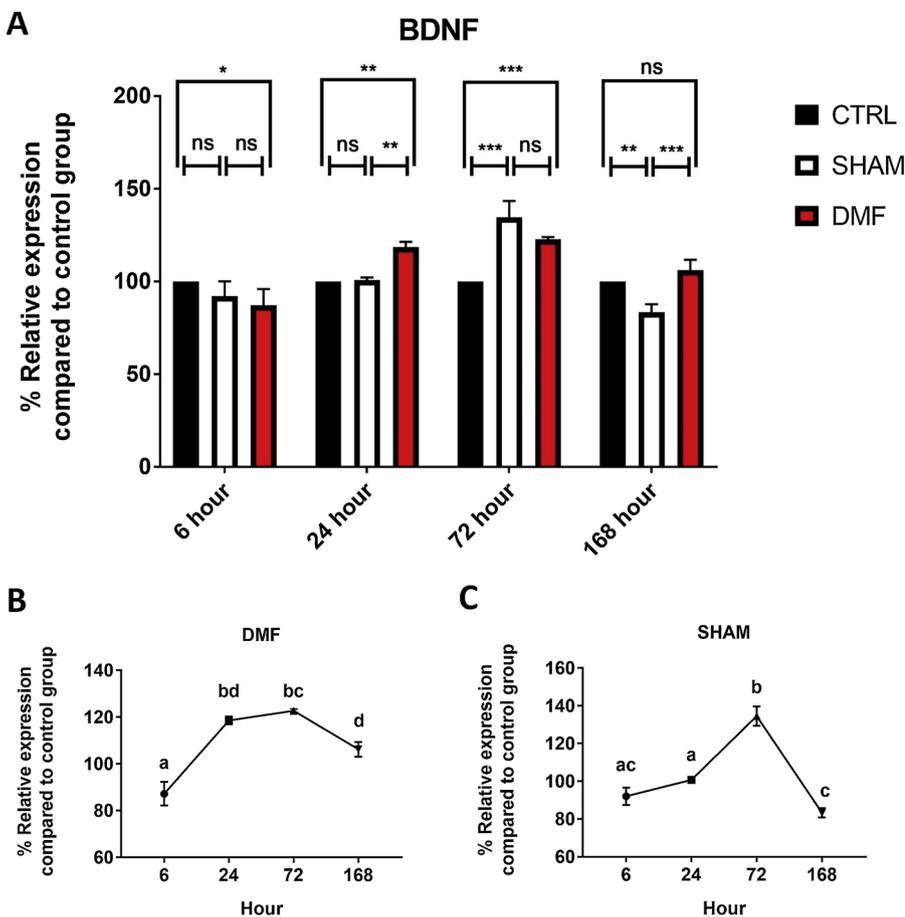
#### 3.4. Effects of DMF on the relative expression of GDNF and VEGF in the EPI-NCSCs

To provide more accurate information about the impact of DMF on other trophic factors, the GDNF and VEGF transcripts were assessed after DMF treatment. In this regard, obtained data revealed the remarkably inhibitory effect of DMF on GDNF mRNA expression in various time points (Fig. 6). On the other hand, no significant changes were detected for VEGF mRNA levels among all time points (Fig. 7).

### 4. Discussion

The chief aim of present study was to assess the effects of DMF on major trophic factors, mainly the ones with neuroprotective effects in the EPI-NCSCs in order to increase their curative potential as new therapeutic modalities for neurological disorders. Here, we evaluated the relative expression of NGF, NT-3, BDNF, GDNF and VEGF in the EPI-NCSCs up to 168 h following incubation with  $10\mu\text{M}$  DMF.

NGF, a member of neurotrophic factors, is essential for the development and maintenance of neurons in the nervous system. This growth factor plays a vital role in the function and survival of cholinergic neurons and has shown to have therapeutic effect on neurological



**Fig. 5.** Relative expression of BDNF mRNA levels in EPI-NCSCs at various time points following treatment. A) Statistical comparison between groups in each time point. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; ns: not significant. B and C): Statistical comparison in each treatment between various time points. Values with different superscripts are significantly different ( $P < 0.05$ ).

disabilities associated with these neurons (Aloe et al., 2012). Since delivering NGF into CNS due to its severe side effects and difficulty to cross the BBB is challenging, using adenoviral neurotrophic gene transfer has been considered as an effective method for delivering NGF into CNS. Also, it is widely reported that transplantation of genetically engineered stem cells that overexpress NGF is an effective approach to cure various neurological diseases (Lee et al., 2012; Marei et al., 2013). In the current study we showed that the relative expression of NGF in the EPI-NCSCs following 72 h treatment with DMF increased by two folds. Therefore, this strategy can be employed to enhance the restorative potential of cell therapy.

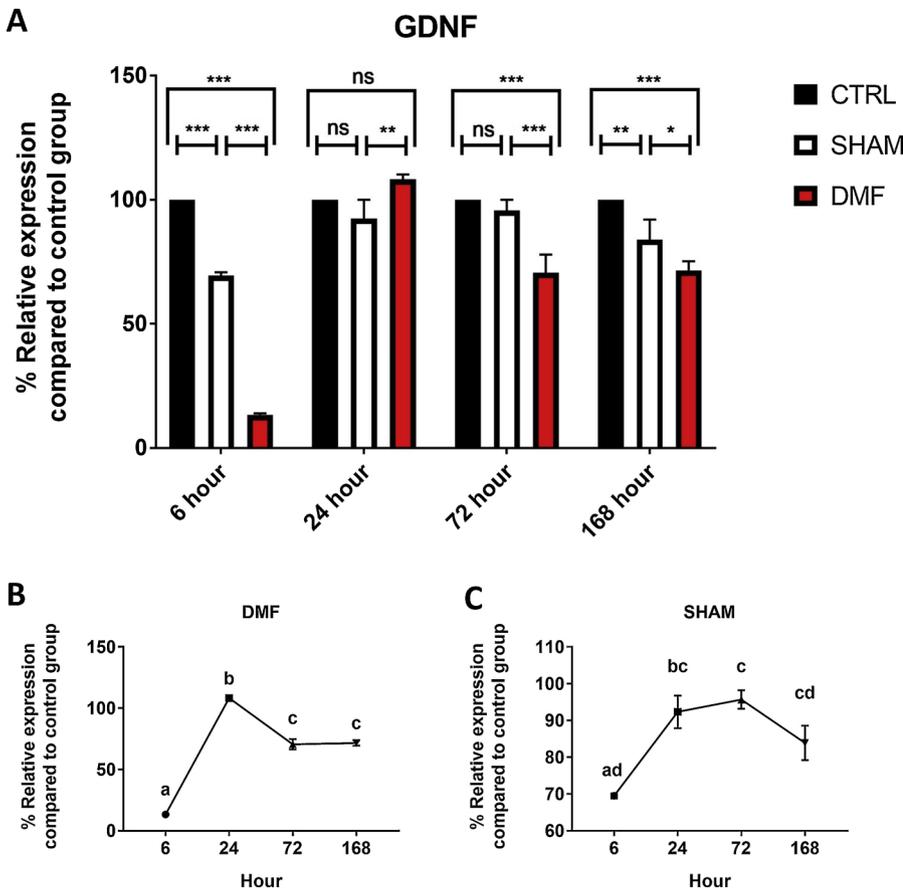
NT-3 is another member of neurotrophic factors family that can prevent mature neurons atrophy and enhance axonal recovery as well as regeneration in the animal model of spinal cord injury (Guo et al., 2007). Furthermore, it has been shown that the regenerative potential of stem cells can be improved when they overexpress NT-3 following genetic manipulation (Boyce et al., 2012; Stewart et al., 2018; Taylor et al., 2006). In the present investigation, we found that three days DMF treatment can up-regulate NT-3 expression in the EPI-NCSCs by more than 800%. Since therapeutic effects of EPI-NCSCs were reported in mouse (Hu et al., 2010; Sieber-Blum et al., 2006), rat (Pandamooz et al., 2018) and dog spinal cord injury (McMahill et al., 2015), pretreatment of this type of adult stem cells with DMF might enhance their curative potential.

BDNF also belongs to the neurotrophin family, which plays a crucial role in cellular functions including neurite outgrowth, axonal elongation, differentiation, maturation, and survival of neurons, neuroplasticity and synaptic modulation (Begni et al., 2017; Garraway and Huie, 2016). There is a growing body of evidence indicating that BDNF-overexpressing stem cells can be employed to ameliorate the devastating condition of various neurological disorders (Deng et al., 2016)

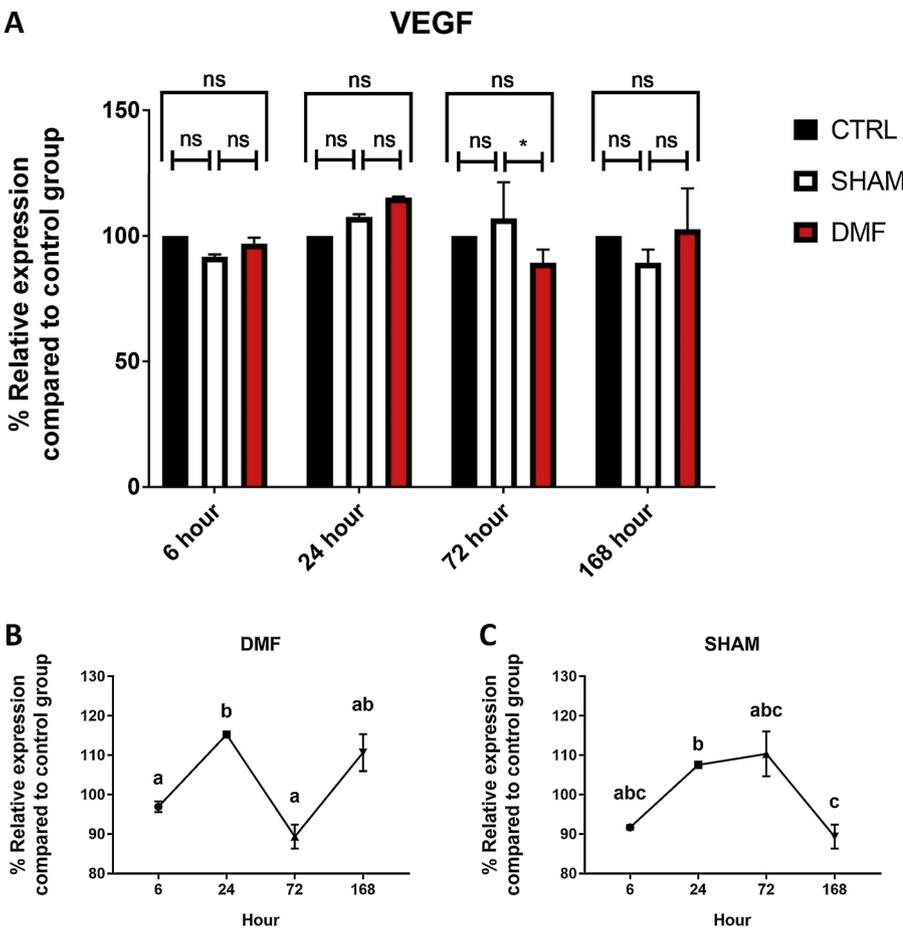
such as animal model of Huntington's disease (Pollock et al., 2016; Wheelock et al., 2016), Alzheimer's disease (Wu et al., 2016), ischemic stroke (Chang et al., 2013; Lee et al., 2010), and spinal cord injury (Khan et al., 2018). Here, we have shown that DMF can also induce BDNF overexpression in the EPI-NCSCs slightly, but significantly following 72 h treatment.

GDNF is a potent promoter of neuronal survival in the peripheral and central nervous system, and its impacts have been widely reported on variety of cells such as cholinergic, serotonergic, noradrenergic and hippocampal neuron as well as autonomic and sensory ganglia and Purkinje cells of the cerebellum (Allen et al., 2013). However, there is contradictory evidence regarding GDNF changing expression following neurological disorders. Here, some investigations showed increased GDNF expression after cerebral ischemia (Duarte et al., 2012); while others, showed decreased expression in Alzheimer's (Budni et al., 2015) and Parkinson's diseases (Chauhan et al., 2001). Our results revealed DMF treatment could reduce GDNF mRNA in the EPI-NCSCs, almost at all-time points after incubation. Hence, this effect should be further evaluated in neurological conditions. Finally, VEGF was the last growth factor assessed in this experiment, which was not affected by 10  $\mu$ M DMF treatment.

Although the exact mechanisms of action through which DMF alters growth factors expression remain to be unknown, several lines of evidence indicate that DMF can exert its beneficial effects via activation of transcription factor nuclear factor-like 2 (Linker et al., 2011) and/or hydroxycarboxylic acid receptor 2 (Chen et al., 2014) pathways. Therefore, the possible mechanisms of DMF action must be investigated in the context of EPI-NCSCs to shed light on how the expression of selected growth factors can change post treatment.



**Fig. 6.** Relative expression of GDNF mRNA levels in EPI-NCSCs at various time points following treatment. A) Statistical comparison between groups in each time point. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ ; ns: not significant. B and C): Statistical comparison in each treatment between various time points. Values with different superscripts are significantly different ( $P < 0.05$ ).



**Fig. 7.** Relative expression of NGF mRNA levels in EPI-NCSCs at various time points following treatment. A) Statistical comparison between groups in each time point. \* $P < 0.05$ ; ns: not significant. B and C): Statistical comparison in each treatment between various time points. Values with different superscripts are significantly different ( $P < 0.05$ ).

## 5. Conclusion

All things considered, successful application of stem cell therapy is likely to depend on the identification of suitable cells that can provide platforms for sufficient expression and secretion of growth factors. Furthermore, if these stem cells positively respond to combination therapy, they would be highly respected. In this regard, EPI-NCSCs therapy in combination with DMF treatment might be considered as a potentially powerful new curative approach for a broad spectrum of neurological disorders.

## Acknowledgments

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