RESEARCH PAPER

Acta Neurobiol Exp 2019, 79: 413–420
DOI: 10.21307/ane-2019-038

Lovastatin alters neurotrophin expression in rat hippocampus-derived neural stem cells in vitro

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Neural stem/progenitor cells hold valuable potential for the treatment of neurodegenerative disorders. The modulation of intrinsic growth factor expression, such as neurotrophins and their receptors, is a necessary step in achieving neural stem cells (NSCs) therapy. The statins have recently been reported to provide both anti-inflammatory and neuroprotective effects. In the developing and mature nervous systems, neurotrophic factors are known to impact neuronal growth and survival. In this study, we investigated for a positive effect of lovastatin on the expression of neurotrophins in the neonatal rat hippocampus-derived NSCs. NSCs were isolated and cultured up to passage three. To confirm cellular identity, immunocytochemical evaluation and flow cytometry analysis were performed using specific antibodies. To determine the optimum concentration of lovastatin, the MTT assay was used. Neurotrophin expression was evaluated using quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR). Flow cytometry results demonstrated that NSCs were positive for nestin, a marker for neural progenitor cells. An increase in cellular viability was observed with a 24 h exposure of lovastatin. Moreover, results showed an increase in mRNA expression for all neurotrophins compared to the control group. Taken together, the results of this study add to the growing body of literature on the neuroprotective effects of statins in neurological disorders. Lovastatin is a promising therapeutic agent for the treatment of neurodegenerative disorders.

Key words: lovastatin, neural stem cells, neurotrophins

INTRODUCTION

Neurotrophins are comprised of a small family of dimeric proteins, which assist in differentiation and survival of peripheral and central neurons. They regulate neurogenesis, synaptic strength, and plasticity (Ivanisevic and Saragovi, 2013), as well as neurons survival, development, and function in the vertebrate nervous system (Vilar and Mira, 2016). Nerve growth factor (NGF), the first described member of the neurotrophin family, is best known for its trophic role in the prevention of programmed cell death in the neuron populations of the peripheral nervous system (Frade and Barde, 1998). In 1982, researchers reported that a second member of the family of neurotrophic factors, brain-derived neurotrophic factor (*BDNF*), exerted a protective effect on the survival of specific dorsal root ganglion neurons, purified from pig brain (Barde et al., 1982). Other members of the neurotrophin family, including ciliary neurotrophic factor (*CNTF*), neurotrophin-3 (*NT-3*), glial cell line–derived neurotrophic factor (*GDNF*), and neurotrophin-4 (*NT-4*), have also been described with distinct trophic effects on different neuronal populations of the peripheral and central nervous system. *CNTF* is broadly studied and known to promote the survival of all classes of neurons (Dubovy et al., 2011). Statins are 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, which are used to treat dyslipidemia and reduce the risks asso-

Received 17 March 2019, accepted 17 September 2019

ciated with atherosclerosis. Statins have recently been considered as potential remedies for the treatment of various neurological disorders and increasing clinical studies are underway (Malfitano et al., 2014). According to recent studies, the use of biomolecules with a positive impact on neurotrophic factors may reduce neuronal damage in neurodegenerative disorders (Lin et al., 2015; Razavi et al., 2015). In this study, we evaluated the effects of lovastatin on the expression of neurotrophins in neural stem cells and found the optimal concentration to induce such effects.

METHODS

Isolation and expansion of NSCs

NSCs were isolated from the hippocampus of three neonatal Wistar rats (5–10 days old) purchased from the Razi Vaccine and Serum Research Institute (Karaj, Iran). Briefly, under deep anesthesia by intraperitoneal injection of ketamine/xylazine (100 mg/kg and 10 mg/kg, respectively), the brains were removed and the dissected hippocampi washed in the ice-cold phosphate-buffered saline (PBS, Gibco), supplemented with 4.5 g/L glucose. The collected tissues were homogenized with a scalpel and then dissociated using a digestion mixture of papain [2.5 U/ml] (Sigma-Aldrich; Germany), dispase II [40 U/ml] (Sigma-Aldrich; Germany) and accutase [1 ml] (Invitrogen; Thermo Fisher Scientific, USA) for 30 min at the room temperature. The cell mixture was passed through a 70 µm cell strainer (Falcon) and centrifuged for 10 min, at 1,000 g and 4°C. Then, the pellets were washed in phosphate-buffered saline. The cells were seeded in non-adherent T25 flasks in NSCs medium containing Dulbecco modified eagle medium/F-12 (DMEM/F12) supplemented with 2% B27 (Gibco; Thermo Fisher Scientific Inc.), 20 ng/ml basic fibroblast growth factor (bFGF; Invitrogen; Thermo Fisher Scientific Inc.), 20 ng/ml epidermal growth factor (EGF; Invitrogen; Thermo Fisher Scientific Inc.), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich; Merck KGaA), and then incubated at 37°C in 5% CO2 to form neurospheres for six days (Pall et al., 2017). The medium was supplemented every two days. In the next step, the cells were dissociated enzymatically using trypsin-EDTA (0.25%; Sigma-Aldrich; Merck KGaA) and mechanically by pipetting to single cells. NSCs (10^5 cells/well) were suspended in DMEM/F12 supplemented with 2% B27, 20 ng/ml bFGF, 20 ng/ml EGF and 3% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific Inc., Waltham, MA, USA) for one week (at 37°C in 5% CO2) in 6-well adherent plates coated with poly-l-lysine and cultured up to passage number three. For identification of these cells, immunocytochemical evaluation, and flow cytometry analysis were performed using anti-nestin monoclonal antibody (ab6142; 1:300; Abcam), followed by incubation with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse antibody 1/300 (Millipore, Billerica, MA, USA, AP307F). Cells were cultured on cover slides and fixed in 3% paraformaldehyde for 20 minutes, followed by a permeabilization step in 100% methanol for 30 min at room temperature. Then cells were incubated with the primary antibody at 4°C overnight, and the next day secondary FITC-conjugated for four hours. Ethidium bromide was used for 30 secs for nuclei counterstaining at room temperature. Images were captured with an Olympus BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan). All experimental protocols were approved by the Zanjan University of Medical Sciences Ethics Committee.

Lovastatin dose-response

The third-passage of NSCs was cultured in 96-well plates (5×10^4 cells/well) in DMEM/F12 medium supplemented with 2% B27, 20 ng/ml bFGF and 20 ng/ml EGF for 24 h. The cells were treated with different concentrations of lovastatin (C24H36O5 – PubChem) (2, 4, 6, 8 and 10 µM) for the next 24 h, which was repeated every 12 h. The NSCs without lovastatin treatment were used as the control group. Then, the cells were incubated with 1 mg/ml MTT (Sigma Aldrich, Germany) solution for four hours. The culture medium was removed and 100 µl dimethyl sulfoxide added to each well to dissolve the formazan crystals. The amount of formazan was quantified at 570 nm using a microplate ELISA reader. The relative cell viability in percentage (Han et al., 2009) was calculated as: Relative cell viability = (A570 of treated samples / A570 of untreated samples) × 100.

Cresyl violet staining

The ability of NSCs to differentiate into neuron-like cells was evaluated by Nissl staining (0.1% cresyl violet). Briefly, the medium was aspirated and the cells were gently washed twice with phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde for 15 min at room temperature. Then, the fixation solution was aspirated and the cell monolayer was gently washed with PBS twice. In the next step, PBS was removed from the cells and staining solution (0.5% cresyl violet) was added, followed by incubation at room temperature for 30 min. After washing with PBS, the cells were observed under light microscope (BX61; Olympus,

Tokyo, Japan) and the blue/violet colored cells were considered positive.

Real-time RT-qPCR

RT-qPCR was carried out using extracted cDNA from control and treatment groups. Total RNA of hippocampal tissues was isolated by TRIzol® (Invitrogen/ Life Technologies). We used 1,000 ng of purified RNA to synthesize 20 µl of cDNA according to a Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, Germany). The cDNA was then used to quantify mRNA levels of the neurotrophins *Bdnf*, *Gdnf*, *Cntf*, *Ngf*, *NT-3* and *NT-4*. Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) was used as the internal control for normalization. RT-qPCR was conducted using primers shown in Table I. The PCR solution contained forward and reverse primers (200 nM each), cDNA (0.5 µl), SYBR® Green I (6.5 µl; Fermentas; Thermo Fisher Scientific, Inc.) and nuclease-free water up to the final volume of 12.5 µl. The PCR reaction was repeated for 40 cycles, each cycle including 15 s in 95˚C followed by 1 min in 60˚C. Relative changes in target mRNA levels were determined using the ΔΔCq method (Livak and Schmittgen, 2001; Mosley and HogenEsch, 2017). The product size of the PCR was later verified by 2% agarose gel electrophoresis. The experiments were repeated three times.

Statistics

Statistical analyses were performed using SPSS software version 15 (IBM; Armonk, New York, United States). All data are presented as the mean ± standard error of mean (SEM) from independent experiments that were repeated three times. One-way ANOVA and Tukey's *post hoc* test were used for data comparisons between the groups. P values less than 0.05 were considered significant.

RESULTS

Neural stem cells culture

The results of the primary culture of NSCs are presented in Fig. 1A-C. The initial culture of NSCs isolated from the neonatal rat hippocampus appeared single and round with clear boundaries in the first 24 h (Fig. 1A). After 3 days, self-renewing neuron-like cells with multipolar processes and growth cone-like features were identified (Fig. 1B). After 3 passages (Fig. 1C) the cells were placed into uncoated 6-well plates to allow neurosphere formation. After 24 h, small spheroids were observed (Fig. 1D). There was a statistically significant difference between groups as determined by one-way ANOVA ($F_{2,27}$=97.26, p<0.001). A Tukey's *post hoc* test revealed that the average spheroid diameter significantly increased after 3 days (136.90 ± 9.94 µm, p<0.001) and 6 days (224.30 ± 11.61 µm, p<0.001) compared to day one (47.40 ± 2.70 µm). There was also a significant difference between day 3 and day 6 (p<0.001) as shown in figures 1D-F and L. Moreover, with higher magnification it was observed that the cells separated from primary spheroids can replicate and form new spheroids (Fig. 1F). The NSCs produced from spheroids were strongly positive for nestin (Fig. 1G and 1H) immunostaining. Flow cytometry confirmed that 77.50% NSCs were positive for nestin (Fig. 1N).

MTT assay

The MTT assay was used to determine the highest tolerated dose of lovastatin for hippocampal derived

Table I. Primer sequences and PCR parameters. Primers for amplification of target sequences and their Gen Bank accession number.

Gene	Gene Accession no.	Sense 5 → 3	Anti-sense 5 → 3
NT-3	NM_031073	GAGGGACCATTCGAGGTGAC	CCAGGGACGTCGACATGAAG
NT-4	NM_013184	ATCAGAGGACCCTGACTTAC	GATACGGTGCTCAGGATAGA
BDNF	NM_001270636.1	TTGAGCACGTGATCGAAGAG	CTCCAGCAGAAAGAGCAGAG
GDNF	NM_019139.1	CGGACGGGACTCTAAGATGA	ATATTGGAGTCACTGGTCAGC
NGF	NM_001277055.1	TCATCCACCCACCCAGTCTTCC	TAATGTTCACCTCGCCCAGCAC
CNTF	NM_013166.1	GAGCAAACACCTCTGACCCT	CAACGATCAGTGCTTGCCAC
GAPDH	NM_017008.4	TTGTCAGCAATGCATCCTGCAC	GTCTGGGATGGAATTGTGAG

Primers were designed by Gene Runner 3.05 software (Product by: info@genfanavaran.com) *NT-3:* Neurotrophin 3; *NT-4:* Neurotrophin 4; *Bdnf:* Brain-derived neurotrophic factor; *Gdnf:* Glial cell line-derived neurotrophic factor; *Ngf:* Nerve growth factor; *Cntf:* Ciliary neurotrophic factor; *Gapdh:* Glyceraldehyde-3-phosphate dehydrogenase.

NSCs. As shown in figure 1M, a gradual increase in concentrations of lovastatin from 6 to 10 µM subsequently decreased cell numbers in the cultures. The highest rate of cell proliferation was observed with 6 µM of lovastatin and a 24 h exposure (81.59% ± 2.44). There was a statistically significant difference between groups as determined by one-way ANOVA ($F_{4,35}$=4.38, p<0.006). A Tukey's *post hoc* test revealed there is significant difference between optimum (6 µM) and 10 µM concentration of lovastatin (63.89% ± 3.09, p<0.002). Lovastatin affected cell viability in a dose-dependent manner, thus we chose 6 µM lovastatin as the optimum dose for further studies to avoid neurotoxic effects of higher concentrations (10 µM). In the time course of the experiment, incubation of NSCs with 6 µM lovastatin for 24 h resulted in the retraction of the cell bodies and processes (Fig. 2A and 2B). Also, the treated cells were positive for Nissl bodies determined by cresyl violet dye (34.66% ± 5.61) (Fig. 2D). As shown in Fig. 2C, no cells were positive for Nissl staining in the control group.

Gene expression

The changes in the mRNA levels of *Bdnf*, *Gdnf*, *Cntf*, *Ngf*, *NT-3* and *NT-4* were examined using RT-qPCR. The results of our study showed that lovastatin increased the expression of neurotrophins relative to the control group with a statistically significant difference between mRNA level-based gene expressions as determined by one-way ANOVA ($F_{5,30}$=22.36, p<0.001) (Fig. 3A). A Tukey's *post hoc* test revealed that mRNA expression levels for *GDNF* significantly increased after lovastatin treatment (128.85 ± 16.71, p<0.001) compared to other genes. The mean fold-changes for *Bdnf*, *Gdnf*, *Cntf*, *Ngf*, *NT-3* and *NT-4* genes were: 34.80 ± 3.23, 128.85

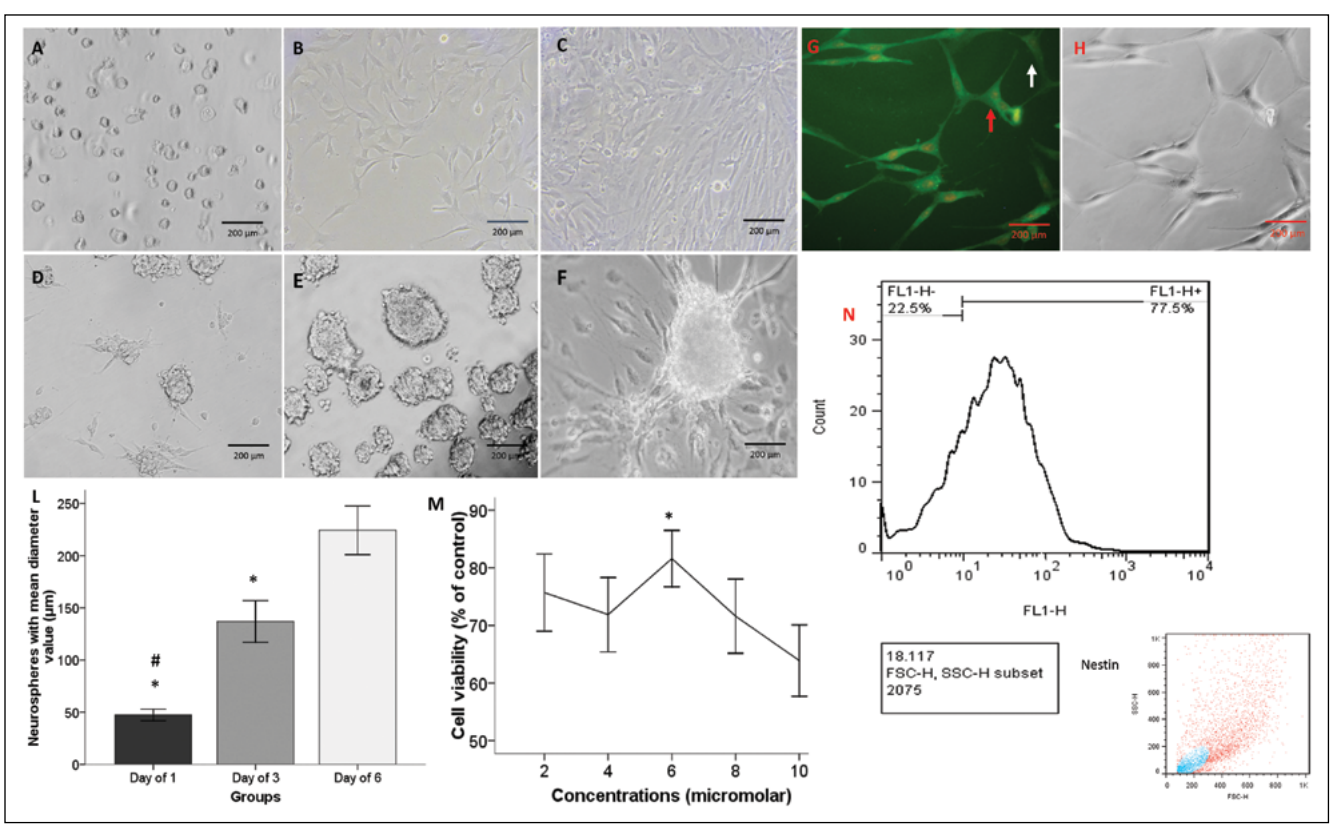

Fig. 1. Representative photomicrograph of hippocampus-derived NSCs, neurosphere diameters and dose-response assay. Cell attachments of the freshly extracted NSCs (A), cells at passage 1 (day 7) (B), and passage 3 (day of 16) (C). (D), (E) and (F) represent floating neurospheres derived from neonate rat hippocampus (rosette like structures) after 1, 3 and 6 days, respectively. (G) represents immunostaining of Nestin (specific markers for neural stem/progenitor cells); the cells were immunostained with relevant primary antibodies and labeled with FITC-conjugated secondary antibody (green color shows positive cells) and the red colors are ethidium bromide counterstaining of the nuclei. (H) represents phase contrast micrographs at the same field. (L) Histogram of neurosphere diameters at different time points; *represents the significant difference with day 6 (*P*<0.001, *post-hoc* Tukey's test); # represents the significance difference with day 3 (*P*<0.001, *post-hoc* Tukey's test). (M) MTT assay graphs represent dose-response NSC viability at different concentrations of lovastatin (results show the mean % viability relative to 0 µM treated NSCs); *represents the significant difference with the 10 µM experimental group (p<0.002, *post-hoc* Tukey's test). (N) represents detection of nestin using flow cytometry analysis. Scale bars 200 µm at × 400 magnification. Each bar represents the average measurement from 5 replicates. The bars indicate the mean ± SEM.

± 16.71, 30.14 ± 3.55, 52.21 ± 6.74, 26.05 ± 3.56 and 42.29 ± 6.31, respectively. The induced NSCs expressed all assessed neurotrophins as detected by gel electrophoresis (Fig. 3B).

DISCUSSION

The results of the present study showed that lovastatin induced expression of neurotrophic factors in the treated NSCs as compared to the control group. To our knowledge, this is the first study to demonstrate the increased release of neurotrophic factors from neural stem cells following lovastatin treatment in-vitro. Neurotrophic factors have been broadly investigated for their roles in supporting survival, proliferation and maturation of neural populations, leading to improved neural regeneration in neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington's diseases (Lin et al., 2015; Xiao and Le, 2016). The effects of neurotrophins are not limited to neurogenesis and axonal sprouting. It has also been shown that neuro-

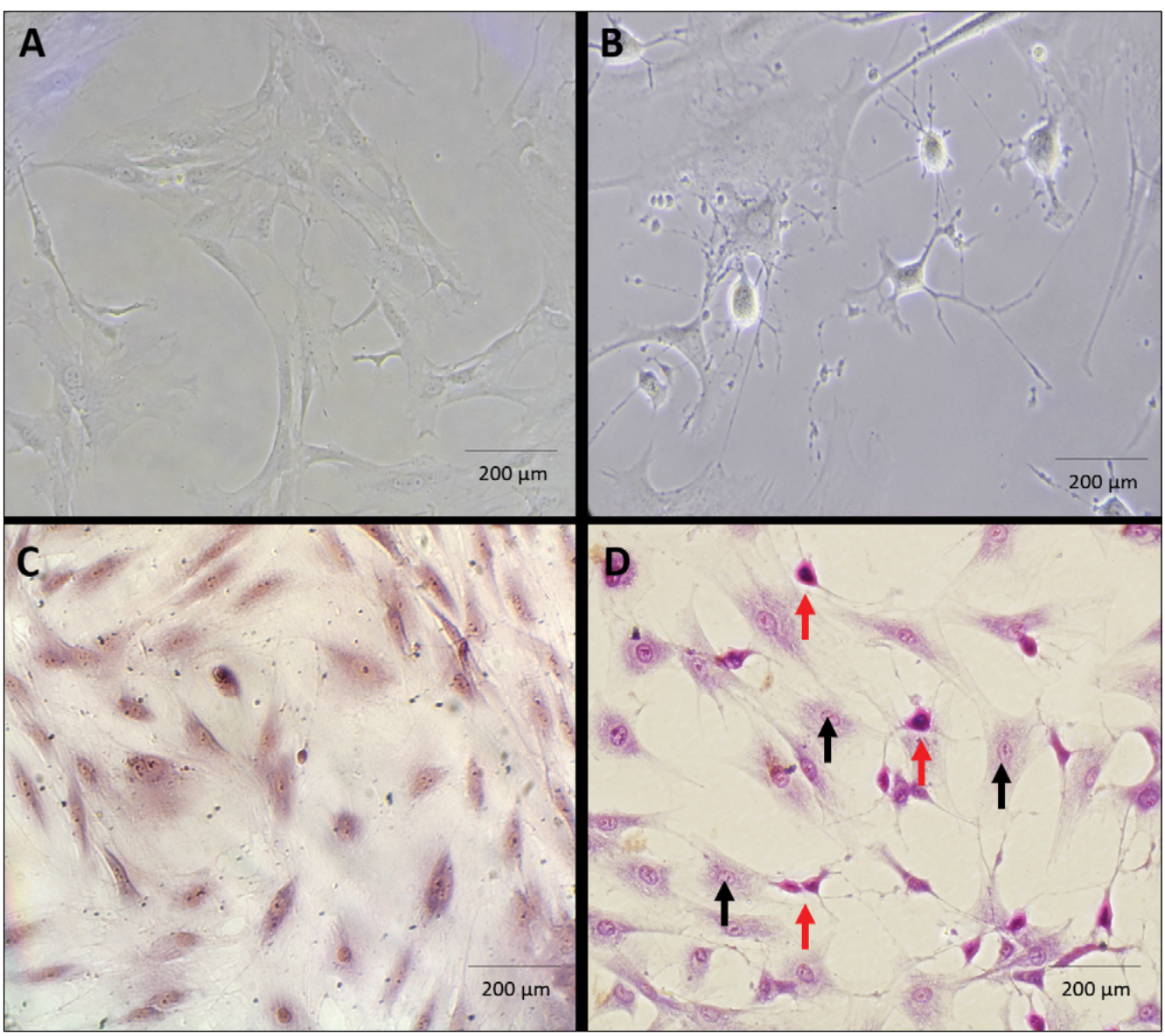

Fig. 2. Phase contrast images of the neuron-like cells differentiation of NSCs using lovastatin. (A) and (B) indicate 0 µM and 6 µM lovastatin-treated NSCs for 12 hours of incubation, respectively. Retraction of cell body and process formation are evident in the 6 µM lovastatin-treated cells. (C) and (D) indicate the Nissl body staining using cresyl violet. (C) negative control (0 µM lovastatin-treated NSCs), (D) positive control (6 µM lovastatin-treated NSCs). Dark blue particles in the cytoplasm show Nissl bodies and the red and black arrows show positive and negative cells, respectively. Scale bars 200 µm at × 400 magnification.

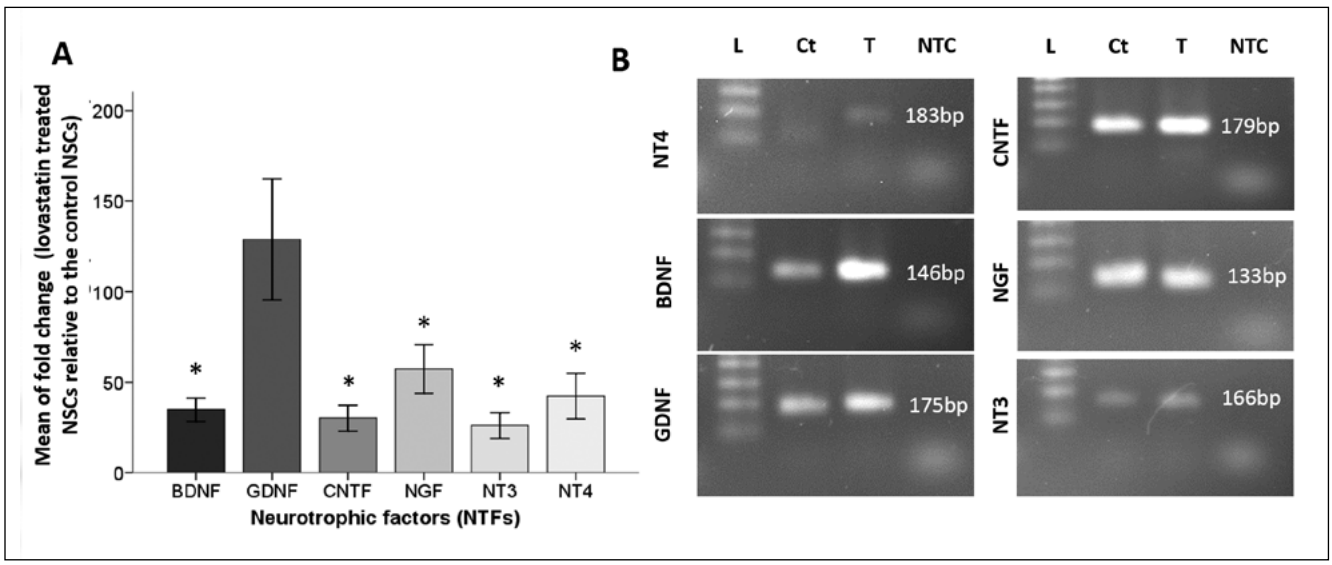

Fig. 3. (A) Quantitative real-time RT-PCR results. All mRNA expression is presented as lovastatin-treated NSCs relative to the control group (non-treated NSCs) normalized to *Gapdh* mRNA amplification. (B) Expression of neurotrophic genes. L, Ct, T and NTC indicate the ladder (100 bp), NSCs treated with 0 μM lovastatin, NSCs treated with 6 μM lovastatin and negative control (without cDNA), respectively. This is a pairwise comparison and the bars indicate the mean ± SEM. *represents the significant difference versus *Gdnf* genes ($p<0.001$, *post-hoc* Tukey's test).

trophins have distinct regulatory effects at various excitatory/inhibitory synapses and for survival of cells in the central nervous system (Quiroz et al., 2010).

Lovastatin, an HMG-CoA reductase inhibitor, has various pharmacological actions, including lowering cholesterol and reducing inflammation, as well as anticancer, antioxidant and, in particular, neuroprotective effects (Nasiri et al., 2016; Yan et al., 2015). This drug has shown neuroprotective activity and easily permeates the blood-brain barrier because of its lipophilicity (Lin et al., 2015). Statins alter the fate of neural stem-progenitor cells (NSPCs) in different ways and during differentiation may lead to the expression of mRNAs through a non-CBP pathway (Carson et al., 2018). Statins also have positive effects on NSPCs by increasing neurogenesis through the Wnt/beta-catenin signaling pathway (Robin et al., 2014). As demonstrated in animals, statins can produce significant toxicity at high doses (Hajar, 2011). In the present study, we demonstrated that increased concentrations of lovastatin lead to decreased cell viability. Other studies have reported that statins affect the G1 phase and alter neural precursor cell divisions in a dose-dependent manner (Carson et al., 2018). Statins are presumed to exert their neuroprotective effects by promoting the release of neurotrophic factors and inducing neurotrophic factor gene expression, such as the BDNF (Roy et al., 2015).

The results of this study simply and clearly add to the understanding of the mechanisms underlying the therapeutic potential of statins in neurological disorders found in previous studies (Rajanikant et al., 2007; Malfitano et al., 2014). Our results showed that the expression of *BDNF* in the treated group increased by approximately 34 fold in comparison with the control group. Most studies have investigated the effect of statins on *Bdnf* expression under in vivo condition. Roy et al. (2015) demonstrated that different statins up-regulate *Bdnf* and NT-3 in neurons, microglia and astrocytes. It has also been reported that treatment of rat primary microglial cultures with 20 μM simvastatin increases *Bdnf* expression (Churchward and Todd, 2014). BDNF supports the survival of existing neurons and encourages the growth and differentiation of new neurons and synapses through axonal and dendritic sprouting (Kim et al., 2017). Chen et al. (2015) reported that neural plasticity was promoted by statin therapy through upregulation of *Bdnf* after stroke in mice. BDNF regulates neuronal survival, cell migration, and synaptic function. Thus, statins, along with increased *Bdnf* expression, can play an important role in improving and restoring brain tissue after stroke (Chen et al., 2005). Han et al. (2011) demonstrated the role of simvastatin on functional improvement after spinal cord injury in rat by upregulating the expression of *Bdnf* and *Gdnf*. It has been suggested that BDNF promotes the survival of subventricular zone neurons and differentiation of postnatal hippocampal stem cells. In our study, the results showed that lovastatin increased *Bdnf* mRNA levels relative to non-treated NSCs. An increase of *Gdnf* mRNA expression was seen in our study. It was reported that simvastatin significantly increased the expression of *Bdnf* and *Gdnf* in the rat model of spinal cord injury

(Gao et al., 2015). GDNF plays an important role in the survival of neurons, in enhancing the remyelination of damaged axons and causes neuronal regeneration after spinal cord injury (SCI) (Razavi et al., 2017).

Several studies have shown therapeutic effects of GDNF in various diseases of the central nervous system, such as Parkinson's disease, stroke and epilepsy (Koppula et al., 2012; Zilliox et al., 2016). In rat models of SCI, the use of statins was associated with improved locomotor activity through up-regulation of *Gdnf*, *Bdnf* and *Vegf* expression (Kahveci et al., 2014). In our study, treatment with lovastatin lead to a significant increase in *Gdnf* mRNA expression. Simvastatin has also recently been demonstrated to improve peripheral nerve regeneration and functional recovery in an experimental model of sciatic damage that involves elevation of levels of *Gdnf* and several other growth factors (Guo et al., 2018). Quantitative PCR results from the present study indicate that lovastatin also increased *Cntf* expression. CNTF is expressed mainly in glial cells as well as in neurons, and its cytoprotective effect appears to be exerted after stress or damage. CNTF has recently been used to treat patients with neurodegenerative diseases such as ALS (Purser et al., 2013). In this study, we found that treating NSCs with lovastatin led to an increase in *Ngf* mRNA expression. It has been reported that statins such as simvastatin and atorvastatin enhance expression of growth factors (BDNF, VEGF and NGF) and activate the PI3K/Akt-mediated signaling pathway after experimental intracerebral hemorrhage (Yang et al., 2012). NGF is critical for the survival and maintenance of sympathetic and sensory neurons. We observed that lovastatin led to an increase in the expression of *NT-3* and *NT-4*, especially *NT-4*. Statins have been reported to dose-dependently up-regulate *Bdnf* and *NT-3* mRNA expression in mouse primary astrocytes (Roy et al., 2015). Statins were also shown to enhance expression of growth factors (BDNF, VEGF, and NGF) and activate the PI3K/Akt-mediated signaling pathway after experimental intracerebral hemorrhage (Yang et al., 2012). NT-3 belongs to the NGF family; it plays an essential role in cell survival, axonal growth and neuronal plasticity (Xu et al., 2002). It helps to support the survival and differentiation of existing neurons and encourages the growth and differentiation of new neurons and synapses. In-vitro studies demonstrated that exogenous NT-3 increased proliferation of neural crest and somite-derived NSCs (Levenberg et al., 2005). Finally, there are limited studies on the role of statins in the alteration of CNTF, NT-3, and NT-4 expression, making it hard to draw conclusions from our results. The most recently discovered member of this family is NT-4, which plays a role in the survival and differentiation of vertebrate neurons (Proenca et al., 2016).

The mechanisms by which neurotrophins and growth factors determine cell fate is still not fully understood. In this study, the flow cytometry analyses showed 77.50% of cells expressing nestin. This result may indicate contamination of primary cells isolated from brain tissue (the possible presence of mature astrocytes and microglia). Astrocytes were cultured under adherent culture conditions and mature astrocytes do not show nestin expression (Cho et al., 2013). The downregulation of nestin expression in astrocytes parallels the increase of glial fibrillary acidic protein (GFAP) in differentiating astrocytes (Cho et al., 2013). One of the limitations of this study is that the cells were not purified but brain cells can release neurotrophins and are sensitive to statin treatment both in vitro and in vivo (McFarland et al., 2014). Statins have been reported to upregulate *Bdnf* and NT-3 in neurons, microglia and astrocytes (Roy et al., 2015). Additionally, due to scientific sanctions against Iran and limited financial resources to conduct this investigation, we did not perform post-transcriptional analysis of the gene products using ELISA with antibodies. As a result of the sanctions, antibodies are difficult to obtain and very expensive.

Encouragingly, statins have been broadly identified as possible preventative or treatment options in a number of neurological disorders, such as stroke, epilepsy, depression, cancers and brain and spinal cord injuries. Results from preclinical animal models suggest that statins may be neuroprotective in response to acute brain injury and chronic neurodegenerative disorders (Roy et al., 2015). Our results, in summary, importantly add to the growing body of evidence on the neuroprotective effects of statins for neurological disorders and support that lovastatin is a promising therapeutic agent for the treatment of neurodegenerative disorders. Future investigations are necessary to validate the conclusions drawn from this study examining the underlying cellular and molecular mechanisms of neurotrophin regulation by statins.

CONTRIBUTOR'S STATEMENTS

Farzaneh Fakheri: Executed the research project; Alireza Abdanipour: Designed the study, analyzed the data, wrote the manuscript and supervised experiments; Kazem Parivar: supervised experiments; Iraj Jafari Anarkooli: advisor; Hossein Rastegar: advisor.

ACKNOWLEDGMENTS

This study was partly funded by Zanjan University of Medical Sciences, Zanjan, Iran (Grant number: A-12-82-14).

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