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Original Article

Acetylsalicylic acid enhanced neurotrophic profile of epidermal neural crest stem cells: a possible approach for the combination therapy

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ABSTRACT

Introduction: Stem cell therapy is considered as a promising strategy to treat neurological disorders. Amongst different cell types that are recruited under these devastating conditions, epidermal neural crest stem cells (EPI-NCSCs) are known as potential candidates. Acetylsalicylic acid (ASA or aspirin) is one of the commonly prescribed drugs that might affect the therapeutic potential of the transplanted stem cells. Hence, the present study aimed to evaluate the effects of ASA on the expression of fundamental growth factors involved in restorative pathways expressed by EPI-NCSCs *in vitro* for possible combination therapy's purpose.

Methods: EPI-NCSCs were obtained from the rat's hair follicle. The appropriate ASA concentration to treat the cells was defined based on the MTT assay and then the obtained cells were treated with 80 or 800µM ASA for 1, 3 or 7 days. The relative expressions of *Bdnf, Gdnf, Ngf, Neurotrophin-3, Vegf, Gfap,* and *doublecortin* were finally assessed by qRT-PCR.

Results: The obtained data revealed that the growth factors expressions are influenced by concentration and duration of the treatment applied. One-day ASA treatment was found to be able to increase the expression of all the evaluated genes, except *Gdnf* and *doublecortin*, which elevated three days later. Herein, seven-day treatment of stem cells with 800µM ASA resulted in higher levels of *Bdnf, Vegf,* and *doublecortin*.

Conclusion: Therefore, combination of aspirin and EPI-NCSCs might increase the therapeutic potential of these stem cells to treat neurological disorders.

Keywords:
Aspirin
ASA
EPI-NCSCs
Growth factors
Neurological disorders

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Introduction

Nowadays, efforts to find therapeutic strategies in patients with neurological disorders, in order to enhance the level of bioactive trophic factors, are strikingly increasing. Stimulating cell survival, regeneration and differentiation are known as the main roles of these trophic factors, particularly the ones that have neurotropic properties. Accordingly, reinforcement of these actions is an attempt to regenerate the damaged tissues (Pöyhönen et al., 2019). In this scenario, stem cells have a capacity to generate the efficient cells, which can consequently replace the damaged cells and/or secrete trophic factors. There is a large body of evidences on the use of stem cells to treat neurological disorders, the majority of which are based on the animal models rather than human ones. Although the effectiveness of stem cell's transplantation to substitute the injured cell is controversial yet, there is no doubt that stem cells are able to increase the neurotrophic and protective factors in the damaged nervous system (Song et al., 2018). Hence, there is a high hopefulness that stem cells can play a crucial role in reducing the tissue damage through increasing the synthesis and releasing growth factors.

It was demonstrated that various elements can influence the therapeutic effects of such treatments, including the culture medium, cell type, cell numbers and transplantation method (Sisakhtnezhad et al., 2017). Moreover, medications consumed by a patient can affect the therapeutic potential of stem cells. Acetylsalicylic acid (ASA), also known as aspirin, is one of the medications commonly prescribed under various conditions like stroke (Divani et al., 2013), which may affect the therapeutic capabilities of the transplanted stem cells. In addition, several experimental studies indicated the effect of aspirin on proliferation and differentiation of various cell types (Hao et al., 2018; Jin et al., 2018).

Epidermal neural crest stem cells (EPI-NCSCs) are multipotent stem cells residing in the bulge region of adult's hair follicle, which possess the advantages of both embryonic and adult stem cells altogether. In addition, they reside in the skin, which makes them accessible with minimal invasive interventions (Sieber-Blum et al., 2006). Since these stem cells are ontologically related to the nervous system, EPI-NCSCs can be considered as promising stem cells for the treatment of various neurological disorders, due to their effectiveness in stroke (Salehi et al., 2020), Alzheimer's disease (Esmaeilzade et al., 2012), peripheral nerve injury (Li et al., 2017) and also in the *in vivo* (Hu et al., 2010; Shalmani et al., 2020) and *ex vivo* (Pandamooz et al., 2016; Pandamooz et al., 2018) models of spinal cord injury.

Considering the widespread prescription of aspirin (Divani et al., 2013) and increasing number of studies employing stem cells to treat neurological disorders (Song et al., 2018), the present study was designed to evaluate the effects of ASA on EPI-NCSCs *in vitro* for possible combination therapy's purposes. In this regard, the expressions of five fundamental growth factors involved in regenerative pathways expressed by EPI-NCSCs were assessed followed by one-week incubation with ASA. In addition, the expressions of neuronal and glial markers were evaluated in the ASA-treated EPI-NCSCs.

Material and Methods

Isolation and culture of EPI-NCSCs

In the present experimental study, to obtain EPI-NCSCs, 3-week old male *Sprague-Dawley* rats (n=10) were purchased from the Center of Comparative and Experimental Medicine, Shiraz University of Medical Sciences (SUMS). This experiment was approved by the Animal Care Committee of Shiraz University of Medical Sciences, Shiraz, Iran (1396-01-67-16182) and the Ethics Committee of Animal Use at this University approved the study procedures (IR.SUMS.REC.1396.S965).

Animals were killed by cervical dislocation. Followed by cutting the whiskers pad under the aseptic condition (Figure 1A), hair follicles were isolated (Figure 1B), then the bulges were micro-dissected and explanted on 4-well collagen-coated culture plate (Figure 1C). Subsequently, the explants were fed by minimum essential medium (α-MEM, Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (Gibco, USA), 5% day-11 chick embryo extract, and 1% penicillin / streptomycin (Gibco, USA), and were then incubated in a humidified atmosphere at 37°C with 5% CO2. When the migration of EPI-NCSCs occurred and confluency reached around 80-90%, the cells were detached with 0.25% trypsin/EDTA (Gibco, USA).

Verification of the migrated EPI-NCSCs

To verify EPI-NCSCs, we performed immunofluorescent staining against nestin, as the neural crest stem

TABLE 1: Primer sequence used in quantitative polymerase chain reaction

Gene	Sequence	Amplicon (bp)
Ngf	F-CCCAATAAAGGCTTTGCCAAGGAC	78
	R-AACAACATGGACATTACGCTATGC	
Nt-3	F-GACACAGAACTACTACGGCAACAG	184
	R- ACTCTCCTCGGTGACTCTTATGC	
Bdnf	F-CGATTAGGTGGCTTCATAGGAGAC	182
	R-AGAACAGAACAGAACAGAACAGG	
Gdnf	F- GCTGACCAGTGACTCCAATATGC	192
	R- CCTCTGCGACCTTTCCCTCTG	
Vegf	F- ACTTGAGTTGGGAGGAGGATGTC	183
	R- GGATGGGTTTGTCGTGTTTCTGG	
Gfap	F- GGGACAATCTCACACAGGACCTC	162
	R- CCTCCAGCGACTCAACCTTCC	
Dcx	F- CGCCGCAGCAAGTCTCCAG	185
	R- TCGCCAAGTGAATCAGAGTCATCC	
Actin, Beta	F-TCTATCCTGGCCTCACTGTC	122
	R-AACGCAGCTCAGTAACAGTCC	

cells' marker, as well as doublecortin (DCX) and β-III tubulin, as immature neurons markers, as described earlier (Pandamooz et al., 2020). Briefly, the migrated cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 1% bovine serum albumin plus 1% normal goat serum, and finally incubated overnight with the following primary antibodies: mouse anti-nestin (#ab6142, abcam, USA), rabbit anti-DCX (#ab77450, Abcam, USA), and rabbit anti- β-III tubulin (#ab18207; Abcam, USA).Ultimately, the cells were incubated with goat anti-mouse IgG Alexa Fluor 488 (#A11001, Invitrogen, USA) or FITC conjugated goat anti-rabbit IgG (#F1262, Sigma-Aldrich, USA) secondary antibodies and then counterstained with DAPI (Sigma, #D9564). Of note, images were taken using an Olympus inverted fluorescence microscope.

Cell viability assay

The MTT assay was employed to determine the cytotoxicity of ASA on EPI-NCSCs. For this purpose, the cells were divided into 3 groups based on the duration of treatment, as well as 9 subgroups based on the concentrations of ASA. Subsequently, they were seeded in 96-well plates and after 24h, the plate medium was replaced with culture medium containing 0, 50, 100, 200, 400, 800, 1600, 3200, and 6400 µM ASA (#A5376, Sigma-Aldrich, USA) prepared in α-MEM containing, 10% FBS, and 1% penicillin/streptomycin. Followed by incubating the cells for 1, 3 or 7 days, the medium was discarded, and 5 mg/ml MTT solution in α-MEM was then added to each well. Subsequently, the cells were incubated for 4 h, and the MTT solution was then replaced with acidic isopropanol, in order to dissolve blue formazan crystals. Finally, the developed color was measured at 570 nm using microplate reader (BioTek, USA).

The evaluation of the target genes expressions

Based on the data obtained from the MTT test as well as from previous studies, 80 and 800µM ASA were selected to investigate the gene expression. EPI-NCSCs were seeded in nine 6-well plates, and after 24 h, the culture medium was replaced with a medium containing 80 or 800µM ASA. The control cells were also cultured in a medium without ASA, and the cells were then incubated for 1, 3 or 7 days. To perform the quantitative real time-PCR, total RNA was extracted from the EPI-NCSCs using the YTzol reagent (Yekta Tajhiz Azma, #YT9063, Iran) in terms of the manufacturer's instructions. Afterward, total RNA concentration was measured by Nan-

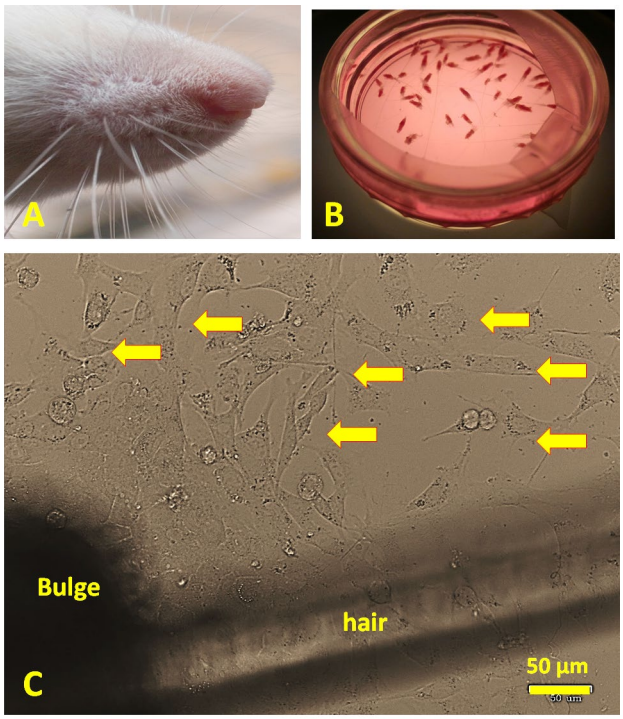

FIGURE 1. Rat whisker pad (A), isolated hair follicles (B) and migrated stem cells from the bulge five days after explantation (C).

oDrop™ spectrophotometer (Thermo Scientific, USA) and treated with DNase I (Thermo Scientific, USA) to remove any trace of genomic DNA. DNase-treated RNAs were employed to synthesize cDNA using a cDNA Synthesis Kit (Yekta Tajhiz Azma, #YT4500, Iran) for reversely transcribing them into cDNA. For analyzing brain-derived neurotrophic factor (*Bdnf*), glial cell-derived neurotrophic factor (*Gdnf*), nerve growth factor (*Ngf*), neurotrophin-3 (*Nt-3*), vascular endothelial growth factor (*Vegf*), glial fibrillary acidic protein (*Gfap*) and *Dcx* mRNA levels, cDNA was added to the reaction mix containing gene-specific primers, as shown in Table 1. Of note, these reactions consisted of RealQ Plus Master Mix Green (Ampliqon, Denmark), as well as an appropriate qRT-PCR primer set. Moreover, the reactions were conducted in triplicates in terms of the manufacturer's protocols using ABI StepOne Real-Time PCR system (Applied Biosystems, USA). The PCR conditions were as follows: 15s at 95°C, and 1min at 60°C for 40 cycles. β -actin was included as an endogenous positive control (housekeeping gene) of the amplification to normalize the amount of cDNA among different samples. The expressions of *Bdnf*, *Gdnf*, *Ngf*, *Nt-3*, *Vegf*, *Gfap* and *Dcx* mRNAs were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Moreover, the PCR products were visualized by ethidium bromide staining on 1% agarose gels.

Statistical analysis

Data analysis was performed using GraphPad Prism (Version 7.03, GraphPad Software, Inc., San Diego, CA). Multiple group comparisons were performed by one-way ANOVA and the Tukey posthoc test. The results are presented as the mean±SEM and P value < 0.05 was considered as statistically significant.

Results

Migration and verification of EPI-NCSCs

By passing a few days from the bulges' explantation, migrated EPI-NCSCs appeared around the bulges (Figure 1C). Expressions of nestin, DCX and β-III tubulin were evaluated by immunofluorescent staining (Figure 2) that confirmed the nature of the migrated cells as epidermal neural crest stem cells.

Effects of ASA on the viability of EPI-NCSCs

The data obtained from the MTT assay indicated that there was no significant difference in cell viability among 50, 100, 200, 400, 800, 1600, 3200, 6400µM ASA compared to the control groups by passing 1, 3 or 7 days from the treatments (Figure 3). It is worth noting that this range of ASA concentrations was selected

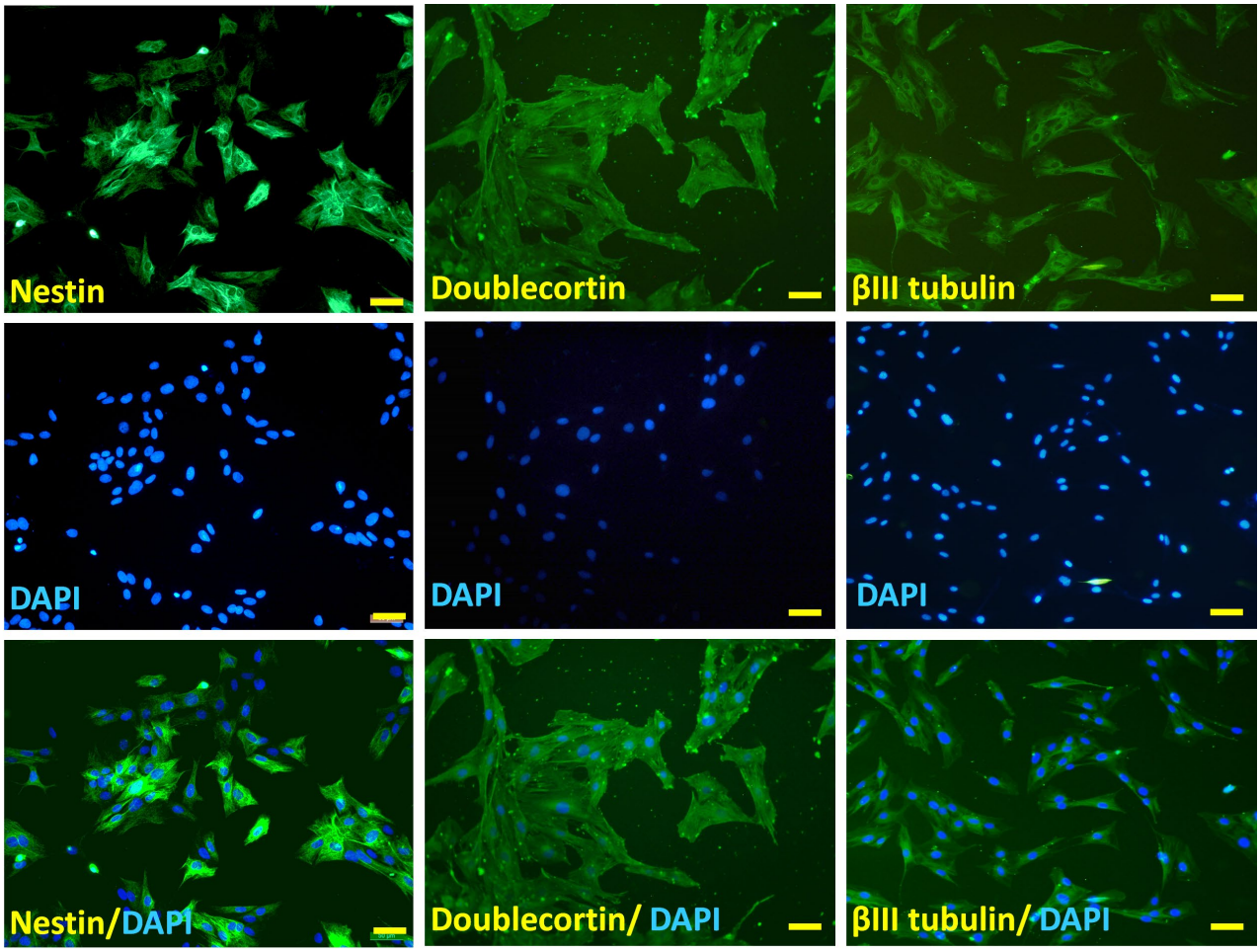

FIGURE 2. Immunofluorescent staining against nestin, doublecortin and βIII tubulin confirmed the identity of migrated cells as epidermal neural crest stem cells. Cell nuclei counterstained with DAPI. Scale bar: 50μm.

based on the previous reports, which evaluated the effect of this agent on different cell types (Dikshit et al., 2006; Wang et al., 2018).

Effects of ASA on the major trophic factors based on the concentration and duration of the treatment

In the current investigation, the epidermal neural crest stem cells obtained from the rat's hair follicle were treated either with 80 or 800μM ASA, and 1, 3 or 7 days later, expressions of five major trophic factors as well as neuronal and glial markers that were already expressed by this type of stem cells, were evaluated.

One-day incubation of EPI-NCSCs with both doses of ASA led to over-expression of *Bdnf*, *Ngf*, *Nt3*, *Vegf* and *Gfap*, while decreased *Gdnf* and *Dcx* expressions (Figure 4). Three days of ASA treatment resulted in up-regulation of *Bdnf*, *Gdnf*, *Ngf*, *Vegf*, *Dcx* and down-regulation of *Nt3*. Notably, at this time point, *Gfap* expression was not significantly affected by the applied treatments (Figure 5). Finally, 800μM ASA treated stem cells for seven days showed higher levels of *Bdnf*, *Vegf*, *Dcx* and lower level of *Nt3's* transcripts. At this time point, 80μM ASA increased *Gdnf* and *Ngf* mRNAs, while 800μM ASA decreased these transcripts compared to the control group (Figure 6).

Discussion

In the present study, the effect of acetylsalicylic acid on the neurotrophic profile of EPI-NCSCs was evaluated in terms of the drug's concentration and duration of the treatment. According to the MTT chemosensitivity assay, ASA did not affect cell viability of EPI-NCSCs in the wide range of 50 to 6400μM. Based on the previously performed studies, different stem cells have different levels of sensitivity to ASA. Accordingly, aspirin have cytotoxic effects on the neural stem cells at a very low

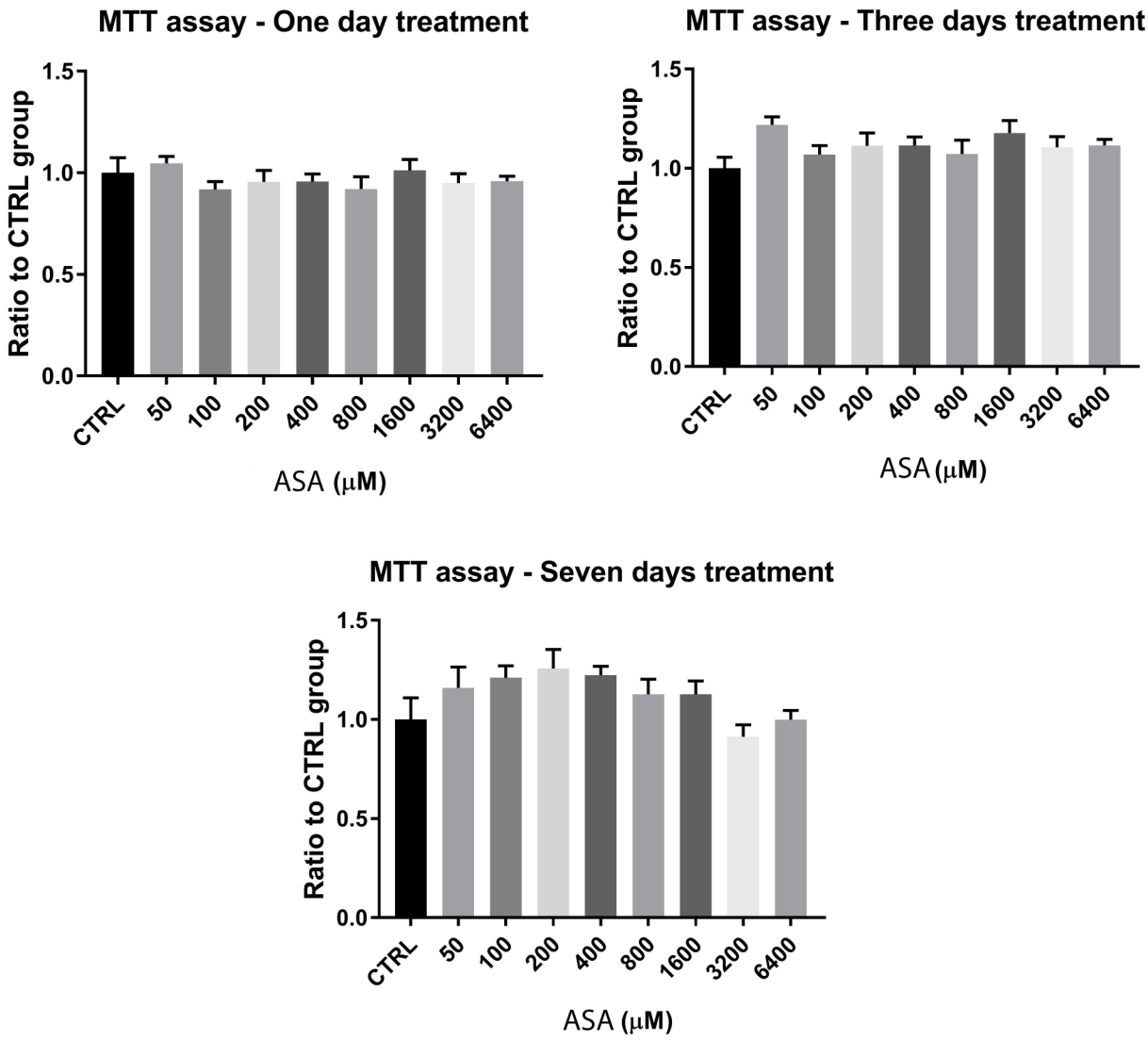

FIGURE 3. Viability of EPI-NCSCs one, three or seven days' incubation with various doses of acetylsalicylic acid (ASA). There were no significant differences between experimental groups.

level of 0.001μM (Hwang et al., 2018). In another study, although 200 or 500μM aspirin did not affect the viability of tendon stem cells, exposure of this types of stem cells to 1000, 2000 or 5000μM of aspirin for 24h led to the morphological apoptosis (Wang et al., 2018). Moreover, Heo et al. (2018) showed that the proportion of apoptotic bone marrow mesenchymal stem cells (BM-MSCs) significantly increased along with increasing the concentration of aspirin. On the other hand, Zhang et al. indicated that co-culturing of the periodontal ligament stem cells with hydrogel loaded ASA could promote cell's proliferation rate at early stage (24 h) with no significant effect at later time points up to day 5 (Zhang et al., 2019a).

Previously, it was reported that the circulating plasma concentration of aspirin in healthy volunteers who consumed aspirin was around 50-300μM (Nagelschmitz et al., 2014). However, different tissues can be exposed to different concentrations of aspirin due to its hydrolysis in biological solutions. In addition, high perfusion tissues such as brain might be exposed to its higher levels (Tatham et al., 2017). Therefore, in the current investigation, we employed 80 or 800μM ASA to treat EPI-NCSCs, which was previously used to treat other cell types (Bhattacharyya et al., 2009; Li et al., 2016). Our findings revealed that ASA could enhance the neuro-

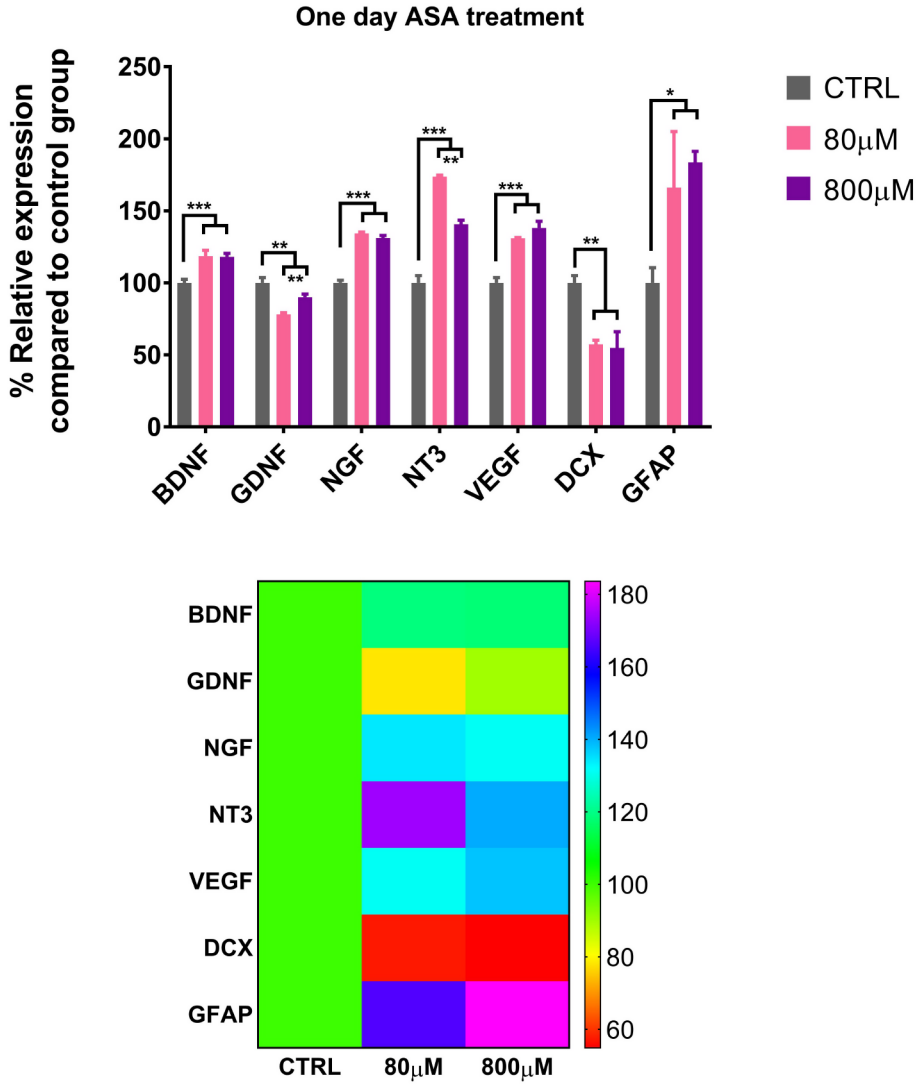

FIGURE 4. (A) Relative expression of brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), vascular endothelial growth factor (VEGF), doublecortin (DCX) and glial fibrillary acidic protein (GFAP) in EPI-NCSCs after one-day incubation with 80 or 800μM acetylsalicylic acid (ASA). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (B) Heat map representation of all evaluated target genes expression in EPI-NCSCs after one-day incubation with 80 or 80μM ASA.

trophic properties of epidermal neural crest stem cells at various time points. Since recovery following a neurological disorder like stroke mostly depends on the triggered neurogenesis in the damaged site (Safari et al., 2019; Owjfard et al., 2020), EPI-NCSCs can be considered as suitable candidates for stem cell-based therapy in such cases due to their high neurotrophic properties (Salehi et al., 2020). These cells not only trigger neurogenesis and angiogenesis, but they can also differentiate into neurons and glial cells (Hu et al., 2006; Gericota et al., 2014).

Patients usually consume various medications; however, their effects on stem cells are still unknown and it seems that these medications can substantially affect the efficiency of stem cells (Marei et al., 2018). Aspirin is one of the commonly used medications, the impacts of which on apoptosis, immune system modulation and osteogenesis were previously studied and demonstrated (Abd Rahman et al., 2016; Wang et al., 2018; Zhang et al., 2019b). In this regard, it was reported that aspirin induces apoptosis in cancer cells, increases the immune modulating factors in bone marrow mesenchymal stem cells and reinforces the osteogenic capabilities of periodontal ligament stem cells (Abd Rahman et al., 2016; Gao et al., 2016; Wang et al., 2018). Tang et al. in 2014 reported that ASA could up-regulate regulatory T cells

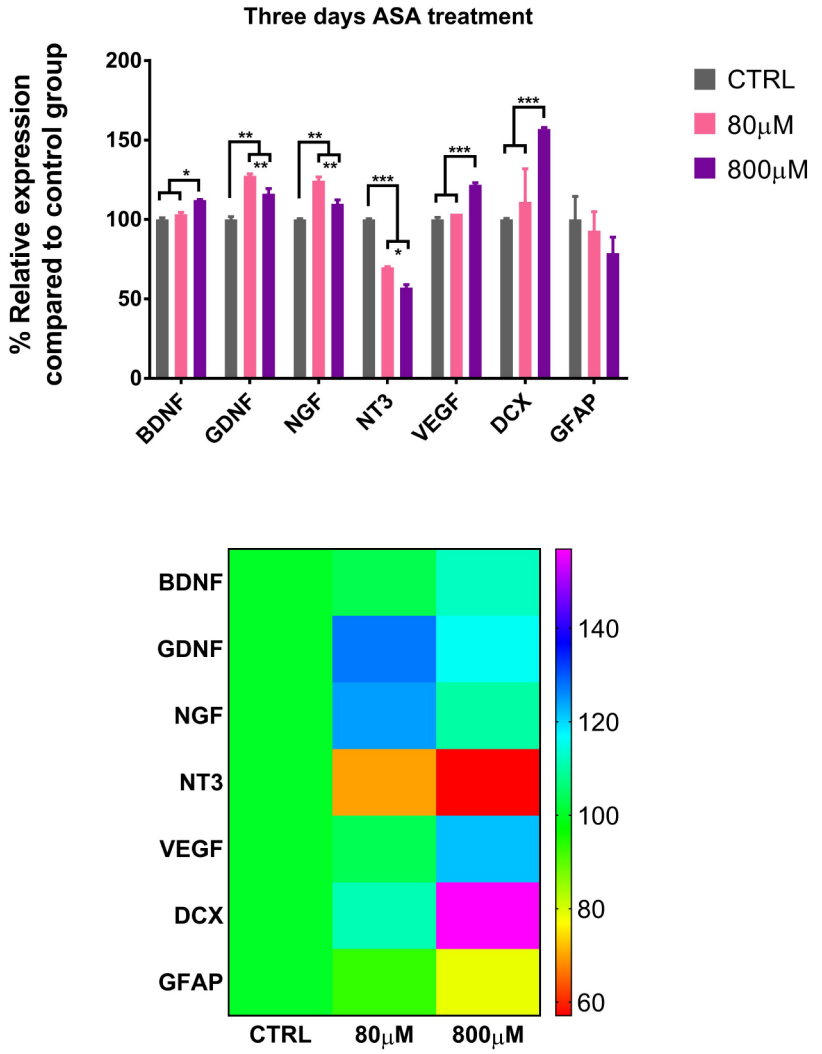

FIGURE 5. (A) Relative expression of brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), vascular endothelial growth factor (VEGF), doublecortin (DCX) and glial fibrillary acidic protein (GFAP) in EPI-NCSCs after three-day incubation with 80 or 800μM acetylsalicylic acid (ASA). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (B) Heat map representation of all evaluated target genes expression in EPI-NCSCs after three-day incubation with 80 or 80μM ASA.

and down-regulate T helper cells along with increasing IL-10 and decreasing IL-17 levels in the BM-MSCs/T cells' co-culture system. Therefore, it has been suggested that ASA treatment can improve the immunomodulatory properties of MSCs *in vitro*. For further confirmation, Khanabdali and colleagues (2018) indicated that aspirin could inhibit the production of the inflammatory markers, IFN-γ and IL-8, and increase the antioxidant capacity of decidua basalis mesenchymal stems.

Nonetheless, limited literature is available on the effect of aspirin on neurotrophins, and in particular their expression in the EPI-NCSCs; however, the effects of valproic acid (Pandamooz et al., 2019; Baharvand et al., 2020), dimethyl fumarate (Salehi et al., 2019) and fingolimod (Pournajaf et al., 2020) on EPI-SCNCs have been recently investigated. The results of the current study show that not only EPI-NCSC viability was not affected following the ASA treatment, but their neurotrophic profile had also increased.

Herein, the obtained data revealed the significant stimulatory effect of 800μM ASA on *Bdnf* mRNA expression at various time points. The 80μM ASA treatment significantly increased the *Bdnf* transcript by passing only one day from the treatment. BDNF is able to induce anti-apoptotic mechanisms and reduce the brain lesion size. In addition, post-injury motor learning has been shown to be associated with the increased BDNF concentration in respective cortices. It was shown that

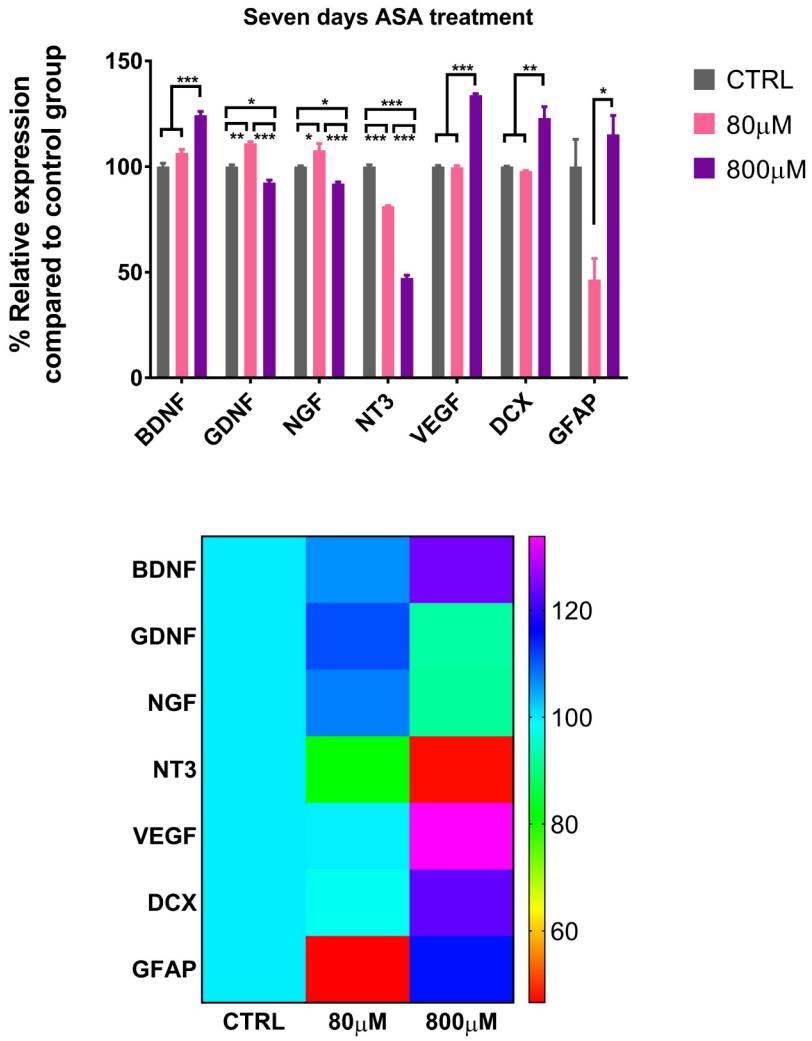

FIGURE 6. (A) Relative expression of brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (A) Relative expression of brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF), neurotrophin-3 (NT-3), vascular endothelial growth factor (VEGF), doublecortin (DCX) and glial fibrillary acidic protein (GFAP) in EPI-NCSCs after seven-day incubation with 80 or 800μM acetylsalicylic acid (ASA). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (B) Heat map representation of all evaluated target genes expression in EPI-NCSCs after seven-day incubation with 80 or 80μM ASA.

BDNF can reorganize the cortical maps through synaptogenesis and increase the formation and deformation of dendritic spines, so it helps neuronal plasticity in many ways (Kellner et al., 2014). Therefore, via increasing *Bdnf* expression in EPI-NCSCs, aspirin could increase the survival, proliferation and differentiation of brain cells as well as the plasticity of the cells under the treatment.

In the current study, experimental doses of ASA led to a significant enhancement of *Ngf* expression up to three days after the treatments; however, after seven days, only 80μM ASA showed a stimulatory effect. Of note, NGF is produced in hippocampus, cerebral cortex as well as pituitary gland, and it is vital for the survival of cholinergic neurons (Aloe et al., 2012). Moreover, it was observed that NGF can be effective on the improvement of cognitive functions (Choucry and Al-Shorbagy, 2019). Recent evidences have indicated that this neurotrophin is able to reinforce angiogenesis and to help in improving neural activity following ischemic stroke (Li et al., 2018). Thus, a combination of EPI-NCSCs and aspirin therapy might improve the cognitive, sensory, and motor functions.

Based on the obtained data, both ASA concentrations significantly elevated the N*t-3* mRNA by passing 24h from the treatments; however, 3 and 7 days later, this effect was reversed and the *Nt-3* expression reduced. NT-3, as a member of the neurotrophin family, plays

crucial roles in the development and function of motor nerves. In addition, this factor plays a critical role in controlling the survival and differentiation of neurons through binding to tropomyosin receptor kinase receptors (P75 NGFR). In the central nervous system, several cell types such as trigeminal mesencephalic nucleus and hippocampus neurons as well as GABAergic cells in the ventral mesencephalon and dopaminergic neurons are responding to NT-3 (Xiao and Le, 2016). Therefore, a combination of EPI-NCSCs and aspirin might improve motor function following performing the needed treatment.

Gdnf and *Vegf* were the other target genes evaluated in this study. Although both doses of ASA intervention resulted in reduced the *Gdnf* transcript one day after the treatments, 80μM ASA elevated the *Gdnf* expression after 3 and 7 days. GDNF plays a vital role in neuronal differentiation during the embryonic development. Due to its neuroprotective properties, this protein protects the function of neurons following any brain damage, so it is considered as a survival factor for dopaminergic neurons. Notably, GDNF dimers via binding to GFRα1 receptors protect neurons from death and ischemic damages (Curcio et al., 2015). As well, our findings revealed that the treatment of EPI-NCSCs with 800μM ASA substantially reinforced the *Vegf* expression in the all evaluated time points. VEGF is a family member of growth factors with critical roles in signaling pathways of vasculogenesis and angiogenesis. This factor also plays an important role in vascular remodeling. Furthermore, VEGFs have neuroprotective properties (Mackenzie and Ruhrberg, 2012). Hence, the combination therapy may increase the capability of EPI-NCSCs in remodeling damaged vessels, and patients might benefit from the neuroprotective effects resulted from this strategy.

Finally, *Gfap* and *Dcx* were the last evaluated genes in the present study. Correspondingly, GFAP is an astrocytic intermediate filament protein, which is almost exclusively found in central nervous system and widely considered as a glial cell marker. Although some previous studies have shown the structural role of GFAP in cytoskeletons and protection against mechanical pressures, the main function of this protein is yet to be identified (Brenner, 2014). The DCX gene encodes a protein called doublecortin, which is involved in cell migration by binding to the microtubule fibers comprising of cytoskeletons. DCX is widely known as a marker of immature neurons (Hua et al., 2008). According to our findings, ASA could up-regulate *Gfap,* while it down-regulated *Dcx* by passing one day the treatment. Notably, three and seven days later, the expression of DCX substantially increased.

The differentiation potential of stem cells after the aspirin induction was evaluated earlier in several studies. As a result, it has been shown that aspirin can induce osteogenic differentiation in periodontal ligament stem cells (Zhang et al., 2019a), dental pulp stem cells (Yuan et al., 2018), mesenchymal stem cells and stem cells from exfoliated deciduous teeth (Liu et al., 2015). Moreover, it was shown that aspirin could promote tenogenic differentiation of tendon stem cells (Wang et al., 2020), odontogenic differentiation of apical papilla (Tanaka et al., 2019) as well as causing cardiomyocyte differentiation in BM-MSCs (Hao et al., 2018). The cultured EPI-NCSCs can also differentiate into various cell types such as glial or neurons (Hu et al., 2006; Gericota et al., 2014). As well, we found that *Dcx* expression increased over time, which its function required additional investigations. Also, further studies are required to evaluate the effects of ASA on the differentiation direction of EPI-NCSCs.

Conclusion

In conclusion, findings of the present study showed that ASA has the potential of reinforcing the neurotropic effect of EPI-NCSCs. In this study, the 800μM ASA elevated the expressions of *Bdnf* and *Vegf* as two key trophic factors involved in restorative pathways in the all evaluated time points. This suggests that therapeutic effects of EPI-NCSCs to treat neurological disorders might be strengthened in the presence of aspirin. However, further *in vivo* studies are needed to clarify the potentials of aspirin and the EPI-NCSCs combination therapy.

Conflicts of interest

The authors have no conflicting interests

Acknowledgment

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