Investigating the synergic effects of valproic acid and crocin on BDNF and GDNF expression in epidermal neural crest stem cells

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Following nerve tissue damage, various events, such as inflammatory responses, microglial activation, endoplasmic reticulum stress, and apoptosis, can occur, which all lead to cell death, prevent axonal growth, and cause axonal circumvolution. So far, several researchers have tended to adopt strategies to reduce the harmful conditions associated with neurological disorders, and stem-cell-based therapy is one of those promising strategies. Epidermal neural crest stem cells (EPI-NCSCs) are a type of stem cell that has widely been employed for the treatment of various neurological disorders. It has been suggested that these stem cells perform their supportive actions primarily through the release of different neurotrophic factors. Hence, in this study, the neuroprotective impacts of valproic acid (VPA) and crocin were evaluated on the mRNA expression levels of brain-derived neurotrophic factor (BDNF) and glial-cell-derived neurotrophic factor (GDNF) in EPI-NCSCs. In this research, we isolated EPI-NCSCs from the hair follicles of the rat whisker pad. Then, the cells were treated with different concentrations of VPA and crocin for 72 h. Subsequently, an MTT assay was performed to define the suitable concentrations of drugs. Finally, real-time PCR was performed to evaluate the mRNA expression levels of BDNF and GDNF in these stem cells. The results of the MTT assay showed that the treatment of EPI-NCSCs with 1 mM VPA and 1.5 mM crocin, and the co-treatment with 1 mM VPA and 500 µM crocin, led to the survival and proliferation of these stem cells. Moreover, the real-time PCR results revealed that both VPA and crocin, both individually and in combination, can significantly increase the expression levels of BDNF and GDNF in EPI-NCSCs. According to the findings of this study, both VPA and crocin, alone and in combination, are potential candidates for enhancing the capacity of EPI-NCSCs to differentiate into neural lineages. Therefore, the co-treatment of EPI-NCSCs with these drugs can be employed for the treatment of various neurological disorders, such as spinal cord injury.

Key words: epidermal neural crest stem cells, valproic acid, crocin, BDNF, GDNF, neuroprotection

INTRODUCTION

Brain-derived neurotrophic factor (BDNF) and glial-cell-derived neurotrophic factor (GDNF) are both related to the neurotrophic factor family, which play essential roles in neural development, cell survival, differentiation, and axonal growth, in both the central and peripheral nervous systems, during develop-ment and in mature systems (Marsh and Blurton-Jones, 2017). During neurodegenerative disorders, such as spinal cord injury (SCI), Alzheimer's disease, and Huntington's disease, neurotrophins can protect, preserve, and rehabilitate neuronal populations (Allen et al., 2013). Until now, various therapies have been considered for the treatment of neurodegenerative disorders, such as cell therapy, molecular therapy, and pharmaceutical

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therapy, but none have been accurate. Hence, researchers began to assess combination therapies for a range of neurological disorders (Neirinckx et al., 2013; Silva et al., 2014).

BDNF is a striking neurotrophin that is broadly expressed in the central nervous system (CNS) (Chen et al., 2013). It was found that BDNF has the capacity to treat neurological disorders (Nagahara and Tuszynski, 2011). When BDNF binds to its receptors, it activates a signaling pathway that leads to neural regeneration and the prevention of inflammatory responses and neuronal apoptosis (Chen et al., 2013). It is notable that BDNF is a vital signaling factor, due to its functions in neurogenesis, neuroprotection, axonal growth, myelination, and synaptic plasticity during various CNS disorders (Harvey et al., 2015). Drugs that can upregulate the expression of BDNF and its downstream effectors, such as the phosphoinositide 3-kinase (PI3K) pathway, the mitogen-activated protein kinase (MAPK) pathway, and the cAMP response element-binding protein (CREB), are suitable options for the treatment of neurodegenerative disorders (Yoshii and Constantine-Paton, 2010; Lin et al., 2016).

GDNF is another neurotrophic factor that is released from glial cells and has several effects that have attracted the attention of researchers for the treatment of neurodegenerative disorders. This neurotrophin activates caspase-3/Akt, which subsequently leads to reductions in astrocyte cell death, the attenuation of microglial activation, and the activation of the PI3K and the extracellular signal-regulated kinase (ERK)-MAPK pathways (Walker and Xu, 2018).

Recent trends in neurotrophic factor research have led to a proliferation of studies that show the benefits of neurotrophic factor overexpression in the human and rodent CNS as a potential therapeutic strategy (Tang et al., 2010; Marsh and Blurton-Jones, 2017).

Cell therapy is one of the appropriate strategies for the treatment of neurodegenerative disorders. Diverse sources and types of stem cells have been studied for use in stem cell therapy to treat neurodegenerative disorders, including neural stem cells, embryonic stem cells, Schwann cells, mesenchymal stem cells, and induced pluripotent stem cells (Martínez-Morales et al., 2013; Silva et al., 2014). Epidermal neural crest stem cells (EPI-NCSCs) have been widely employed for the treatment of various neurological disorders, such as spinal cord injury (SCI). These cells are multipotent stem cells with a high level of plasticity. Although these cells originate from the ectodermic layer (Sieber-Blum and Hu, 2008), they can differentiate into both endodermal and ectodermal cells, such as neurons, Schwann cells, glia, sensory neurons, endocrine cells, smooth muscle cells, and pigment cells, and tissues, including

bone, cartilage, and the connective tissue on the face and ventral neck (Sieber-Blum et al., 2004). EPI-NCSCs can be obtained through a minimally invasive procedure and can be expanded into a pure population of cells through cell culture techniques. Although these stem cells present strong migration abilities, they are not tumorigenic and can be used as autografts (Sieber-Blum and Hu, 2008).

Valproic acid (VPA) is a well-known drug used to treat a wide range of mental and neurological disorders, such as epilepsy, bipolar disorder, depression, schizophrenia, and migraine (Chateauvieux et al., 2010). VPA, a short-chain fatty acid, has attracted considerable interest, due to its neurogenic and neuroprotective effects during SCI treatment, through the inhibition of histone deacetylase (HDAC) and glycogen synthase kinase-3 (GSK-3). Many studies have emphasized the potential role of VPA as an HDAC inhibitor that can upregulate several neurotrophic factors (Jung et al., 2008; Lv et al., 2012; Almutawaa et al., 2014). Because VPA can also inhibit GSK-3, VPA can enhance neuroprotective effects either directly or indirectly, through the Wnt/β-catenin, PI3K/Akt, and MAPK/ERK (MEK) pathway, during psychiatric and neurodegenerative disorder, such as bipolar disorder and Alzheimer's disease. In addition, VPA can trigger a cascade of events that neutralize and compensate for the events associated with secondary damage during SCI, through neuroprotective effects and neurogenesis, via HDAC and GSK-3 inhibition (Chu et al., 2015; Li et al., 2017). During a neural condition, such as SCI, cell damage occurs due to microglial activation, the production of various inflammatory cytokines, and the activation of the nuclear factor (NF)-kB signaling pathway, which produces NF-kB proteins that bind to gene promoters in response to neuroinflammation. This signaling pathway is an important pathway associated with secondary damage during SCI. Hence, VPA, due to HDAC inhibition, can maintain NF-kB in its acetylated state (inactive), which can subsequently suppress the activation of microglia and consequent inflammatory responses (Chen et al., 2018).

Crocin is a primary component in saffron (*Crocus sativus L.*) extract. Crocin is thought to be a useful candidate for the treatment of neurodegenerative disorders because of its anti-inflammatory, anti-apoptotic, and antioxidant effects, which can reduce neuroinflammation and cell death during neurodegenerative disorders (Wang et al., 2015; Yorgun et al., 2017).

This study was designed to evaluate the effects of two neuroprotective agents, VPA and crocin, on the mRNA expression levels of BDNF and GDNF in EPI-NCSCs.

Our study provided the first evidence for the potential use of VPA and crocin as a co-treatment in

EPI-NCSCs to regulate the mRNA expression levels of two neurotrophic factors, BDNF and GDNF.

Overall, based on the wide range of studies that have assessed the impacts of various drugs on EPI-NCSCs (Salehi et al., 2019), in the current investigation, we evaluated the synergistic effects of VPA and crocin on BDNF and GDNF expression levels in EPI-NCSCs, in an *in vitro* context. This therapeutic approach may subsequently ameliorate conditions associated with neurodegenerative disorders and may ultimately be employed in *in vivo* models of neurodegenerative disorders.

MATERIALS

Epidermal neural crest stem cell isolation and culture

In this study, Wistar rats (3-4 weeks old) were sacrificed by cervical dislocation and the whisker pad follicles were dissected and the bulges were rolled out from the hair follicle capsules, as described previously (Sieber-Blum et al., 2004; Pandamooz et al., 2013, 2016). EPI-NCSCs, 3-4 days after bulge explantation, were migrated from the bulge region of the hair follicles into collagen-coated plates (Fig. 1). The culture medium consisted of 85% α-minimum essential medium (α-MEM, Bioidea, Iran), 10% fetal bovine serum (FBS, Bioidea, Iran), 5% day-11 chick embryo extract (CEE, purchased from Shahid Beheshti University), and penicillin/streptomycin (100 U/ml, Bio-idea, Iran). The migrated EPI-NCSCs were incubated at 37°C, in the humidified atmosphere of a 5% CO_2 incubator (Hu et al., 2006).

Cell viability test

To determine the non-toxic doses of VPA (Darou Pakhsh Pharma. Chem. Co, Iran) and crocin (Puyesh Darou Sina, Iran), an MTT assay was performed. EPI-NCSCs were seeded in 96-well plates, at a density of 5×10^3 cells/well, 24 h prior to treatment. Then, EPI-NCSCs were treated with various concentrations of VPA (0.1, 0.5, 1, 2, 5, 7, and 10 mM), crocin (12.5, 50, 100, 500, 1,000, 1,500, 2,000, and 2,500 μM) or both of VPA (1 mM) and crocin (12.5, 50, 100, 200, 500, 1,000, 1,500, 2,000, and 2,500 μM), for 72 h. Each treatment was repeated at least three times. After 72 h of treatment, an MTT solution (125 μg/ml, Sigma, USA) was added to the treated cells, for 3 h, at 37°C. The medium was then aspirated, the formazan product was dissolved in dimethylsulfoxide (DMSO, Merck, Germany), and the absorbance was read at 570 nm, using an iMark™ Microplate Absorbance Reader (Bio-Rad, USA).

RNA extraction, cDNA synthesis, and Real-time PCR

In this study, the effects of VPA and crocin, both alone and in combination, on the transcription levels of GDNF and BDNF in EPI-NCSCs were evaluated by real-time PCR. EPI-NCSCs were seeded at a density of 20,000 cells/well in four 6-well plates. On the first day, the cells were treated with VPA (1 mM), crocin (1.5 mM), or both VPA (1 mM) and crocin (500 μM), while a control group received drug-free medium. On the third and fifth days of the experiment, the entire medium in each well was renewed with fresh medium containing the appropriate drugs. On the seventh day, total RNA was extracted from both control and treated groups, using a total RNA extraction kit (Parstous Biotechnology, Iran), according to the manufacturer's instructions. Then, the RNA concentrations were determined using a Nanodrop system (Thermo Scientific, USA), and afterward, cDNA was synthesized from the extracted mRNA for each sample, using an easy cDNA synthesis kit (Parstous Biotechnology, Iran), with the aid of oligo dT primers. Finally, to compare the expression levels of target genes between the treated groups and the control
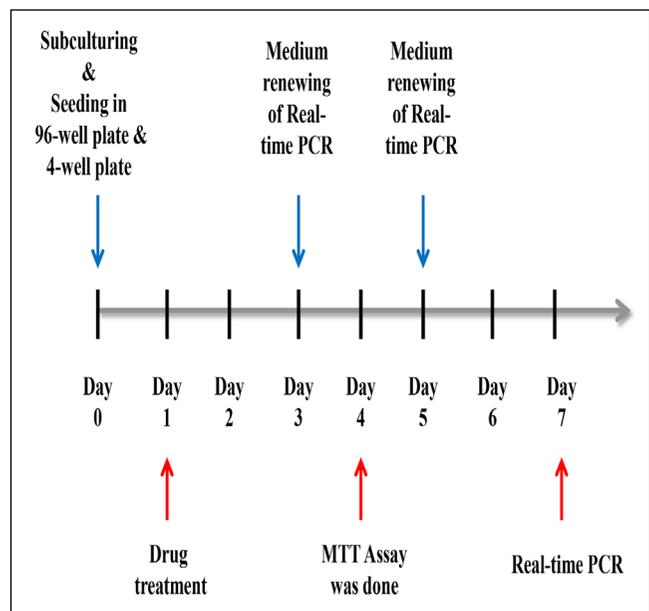
Fig. 1. Timeline scheme. First, EPI-NCSCs were seeded in 96- and 6-well plates. On the first day, cells were treated with VPA, crocin, or both VPA and crocin, and a control group received a drug-free medium. On the third and fifth days, the entire medium in each well was renewed with the appropriate drug-containing medium. On the seventh day, total RNA was extracted from both control and treatment groups, cDNA was synthesized using the mRNA extracted from each sample, and real-time PCR was performed.
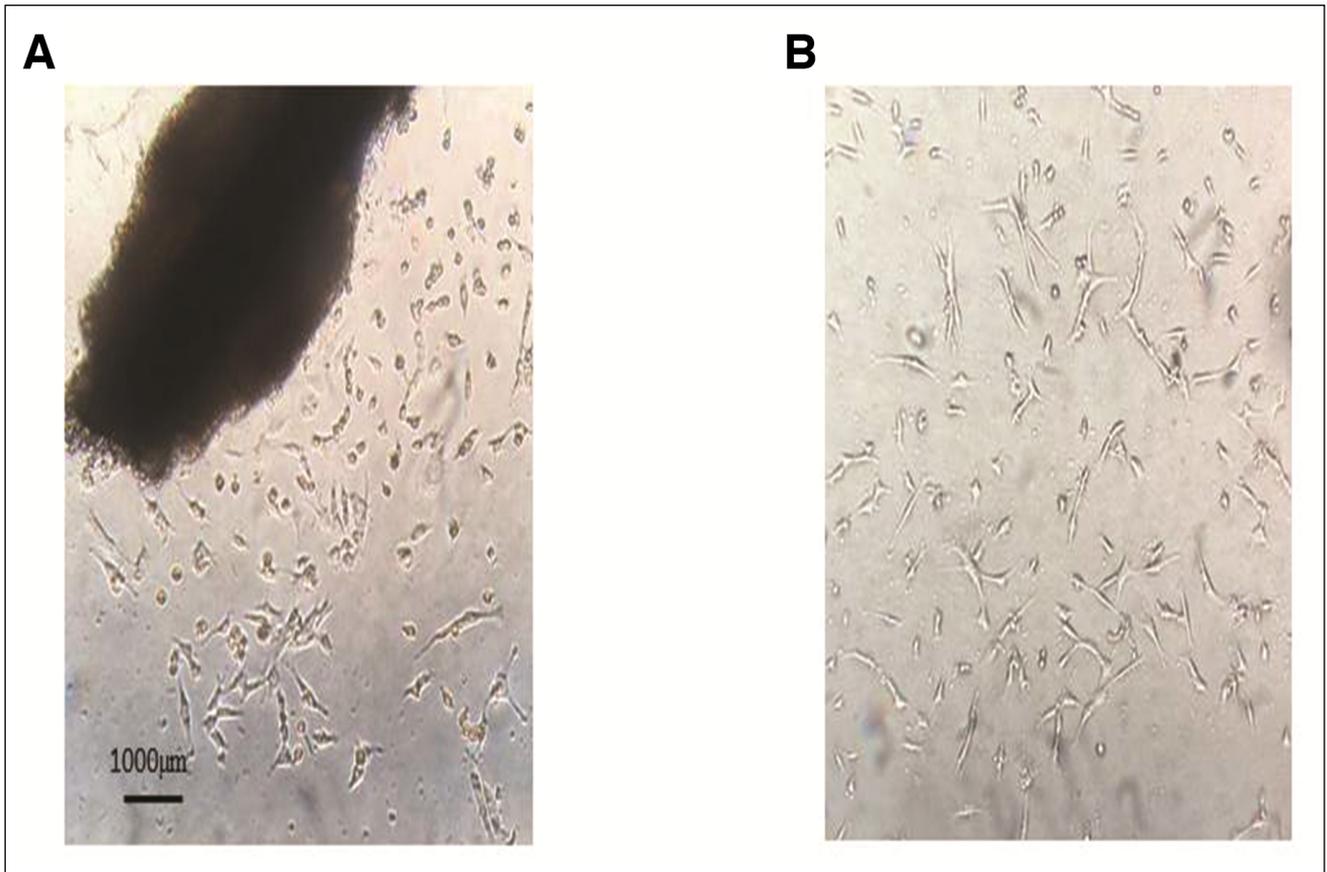
Fig. 2. Migration of epidermal neural crest stem cells from the bulge area. A) Two to three days post explantation of the hair follicle bulge area, EPI-NCSCs migrate from the bulge niche. B) The expansion of these stem cells occurs under *in vitro* conditions, and following 7 days in culture, these migrated stem cells form a pure colony of stem cells (scale bar: 1000 µm).

group, real-time PCR was performed using a SYBER Green PCR master mix (Takara, Japan), in a Corbett research rotor-gene RG6000 Real-Time rotary analyzer PCR machine (Corbett, Australia) (Fig. 2). Finally, the results were normalized against the expression level of a housekeeping gene, beta-actin (β-actin). The specific forward (F) and reverse (R) primer (5′→3′) sequences used for real-time PCR are listed in Table I. Data were analyzed using the ΔΔCt method (Livak and Schmittgen, 2001).

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using either an independent Student's *t*-test independent or a one-way analysis of variance (ANOVA), followed by *post hoc* comparisons. The Student's *t*-test was employed to compare the differences between two groups and the one-way ANOVA was employed to compare more than two groups. Differences between

Table. I. List of primers.

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	Amplicon length (bp)
BDNF	CGATTAGGTGGCTTCATAGGAGAC	CAGAACAGAACAGAACAGAACAGG	182
GDNF	GCTGACCAGTGACTCCAATATGC	CCTCTGCGACCTTTCCCTCTG	192
β-actin	TCTATCCTGGCCTCACTGTC	AACGCAGCTCAGTAACAGTCC	122

groups were considered significant at p<0.05 (*), p<0.01 (**), and p<0.001 (***).SPSS software 22.0 was used for statistical analyses.

RESULTS

In vitro expansion of EPI-NCSCs

The isolation of EPI-NCSCs and their *in vitro* expansion were performed according to procedures previously described by our research group (Hu et al., 2006; Pandamooz et al., 2016, 2017). EPI-NCSCs migrated 2–3 days after explantation from whisker bulges, presenting with stellate morphology (Fig. 2A and 2B). The proliferation of EPI-NCSCs, in culture medium containing α-MEM, 10% FBS, and 5% CEE, was examined daily prior to sub-culturing using an inverted microscope (Pandamooz et al., 2016).

Cell viability

In the current investigation, the MTT assay was performed to define the concentrations of VPA and crocin that did not cause significant decreases in cell survival and did not enhance cell proliferation compared with the non-treated control group. EPI-NCSCs were treated with various concentrations of VPA and crocin for 72 h. The obtained data revealed that none of the tested VPA concentrations (0.1, 0.5, and 1 mM) caused significant cell death, and cell viability was similar to that for the control group. According to the results of the MTT assay, concentrations of 2, 5, and 7 mM VPA decreased cell survival by approximately 40% (p<0.05), and the 10 mM concentration decreased survival by approximately 60% (p<0.001) and demonstrated significant cytotoxic effects in EPI-NCSCs. According to the aim of this study, the proper VPA dose for the treatment of EPI-NCSCs was determined to be 1 mM, a concentration at which cell viability impacts were similar to those observed for the control group, with no significant effects on cell death or cell proliferation; therefore this dose was selected to define the effects of VPA on the mRNA expression levels of the selected trophic factors. In addition, the assessment of crocin showed that concentrations of 12.5, 50, 100, 500, and 1,000 μM promoted cell proliferation by approximately 125%, 123%, 122%, 117%, and 109%, respectively, compared with the control group. In contrast, 2,000 and 2,500 μM crocin had cytotoxic effects in EPI-NCSCs (p<0.001). Moreover, the 1,500 μM concentration of crocin was determined to be the appropriate dose for the treatment of EPI-NCSCs because it was the most effective dose and had no cytotoxic effects. The results of combination
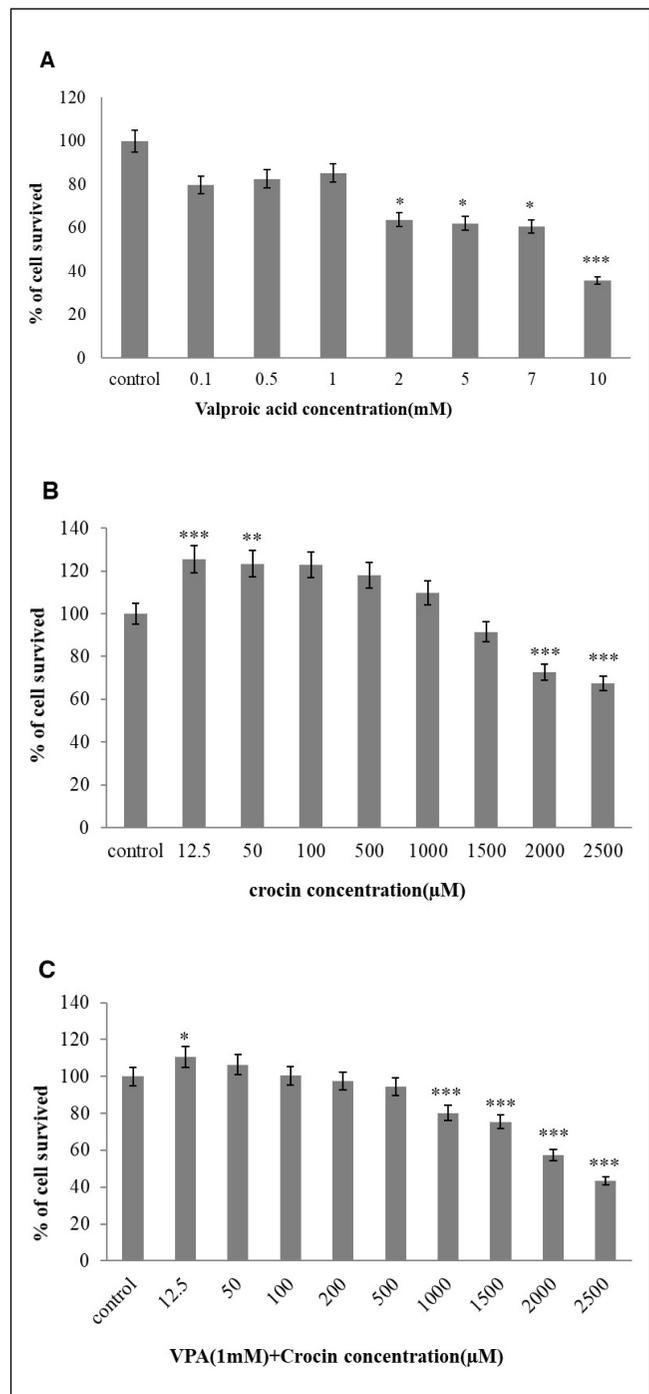
Fig. 3. Investigating the effects of various concentrations of VPA and crocin, both alone and in combination, on the cell viability and proliferation of EPI-NCSCs. A) EPI-NCSCs were treated with different concentrations of VPA, ranging from 0.1 to 10 mM. B) The effects of different concentrations of crocin on these stem cells were defined using the MTT assay. C) Moreover, these stem cells were treated with a combination of VPA and crocin, using a VPA concentration of 1 mM and crocin concentrations ranging from 12.5 to 2,500 μM. Co-treatment with VPA (1 mM) and crocin, at different concentrations, showed that VPA (1 mM) combined with 500 μM crocin was an appropriate dose, which does not cause cell death or proliferation. Values are mean ± SEM (n=3), p<0.001***, p<0.01**, p<0.05*.

treatments, using VPA (1 mM) and different doses of cro-cin, showed that 1,000, 1,500, 2,000, and 2,500 µM crocin decreased cell survival significantly (p<0.001) compared with the control group. As a result, 500 µM was chosen as the appropriate dose for use during co-treatment be-cause this dose resulted in approximately 90% cell viabil-ity, which was appropriate for our scope (Fig. 3).

Effects of VPA on BDNF and GDNF mRNA expression levels in EPI-NCSCs

Following the treatment of EPI-NCSCs with a 1 mM concentration of VPA for 7 days, a significant increase in the BDNF mRNA expression level (p<0.001) was observed compared with the control sample. The treatment of EPI-NCSCs for 7 days with VPA (1 mM) also increased the GDNF mRNA expression level, but this increase was not significant compared with the control group (Fig. 4). Our study showed that renewing the whole medium on the third and fifth days enhanced the effects of VPA on BDNF mRNA expression levels compared with the results observed in a study where the medium was not renewed (Pandamooz et al., 2017).

Effect of crocin on BDNF and GDNF mRNA expression in EPI-NCSCs

The gene expression analysis of the BDNF mRNA ex-pression level showed a significant increase (p<0.001) in EPI-NCSCs following treatment with 1,500 µM cro-

cin compared with that in the control group, but no significant increase was observed for the GDNF mRNA expression level (p>0.05) (Fig. 5).

Synergistic effects of VPA and crocin on neurotrophic factor mRNA expression levels in EPI-NCSCs

In this study, the combination treatment of VPA (1 mM) and crocin (500 µM) for 7 days was examined. In EPI-NCSCs co-treated with VPA and crocin, the mRNA expression levels of BDNF and GDNF significantly in-creased, as quantified by real-time PCR, compared with those following treatment using either VPA or crocin alone (p<0.001). In addition, the GDNF mRNA expression level increased after co-treatment with VPA and crocin (p<0.05), whereas treatments using either VPA and cro-cin alone had no significant effects on GDNF mRNA ex-pression levels (Fig. 6A, B).

DISCUSSION

In this study, we investigated, for the first time, the effects of crocin, alone and in combination with VPA, on EPI-NCSCs by evaluating the mRNA expression levels of BDNF and GDNF. We also showed that co-treatment with VPA and crocin additively enhanced the mRNA ex-pression levels of BDNF and GDNF.

Researchers found that both neurotrophic factors, BDNF and GDNF, are important for the survival, main-
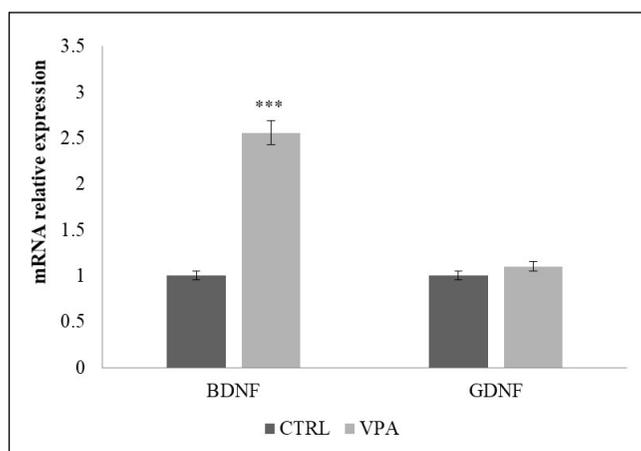
Fig. 4. mRNA expression levels of BDNF and GDNF in EPI-NCSCs treated with VPA for 7 days, during which the medium was renewed twice. Here, the impact of treatment with 1 mM VPA on the mRNA expression level of BDNF in EPI-NCSCs was defined by real-time PCR. The influence of this drug was also assessed for the expression level of GDNF mRNA. Values are mean ± SEM (n=3), p<0.001***, p<0.01**, p<0.05*.
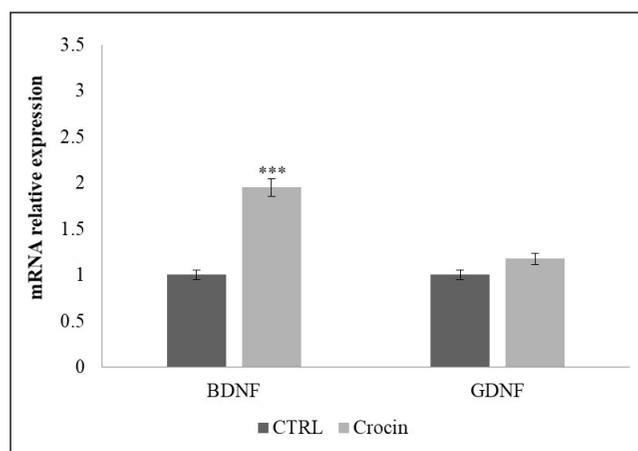
Fig. 5. mRNA expression levels of BDNF and GDNF in EPI-NCSCs after treatment with crocin for 7 days, during which the medium was renewed twice. Crocin can significantly increase the expression level of BDNF mRNA in EPI-NCSCs compared with the control group. The expression level of GDNF did not elevate significantly following this treatment compared with that in the control group. Values are mean ± SEM (n=3), p<0.001***, p<0.01**, p<0.05*.
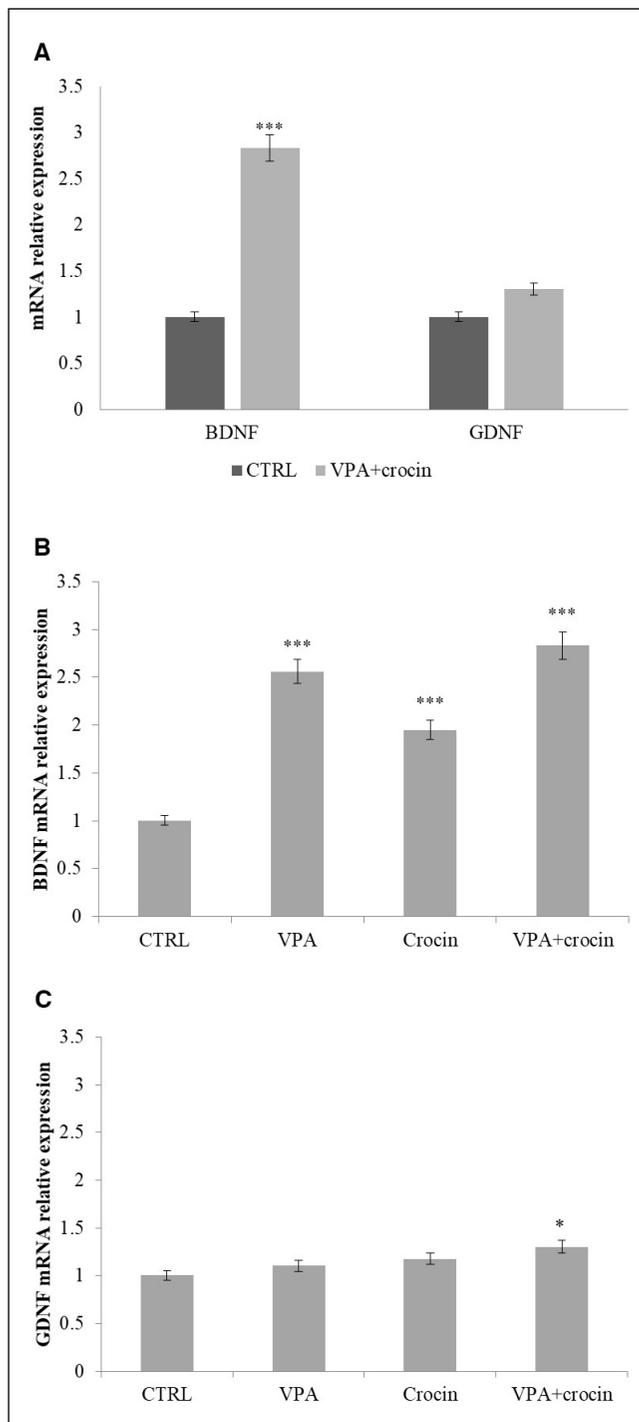
Fig. 6. mRNA expression levels of BDNF and GDNF in EPI-NCSCs treated with a combination of crocin and VPA for 7 days, during which the medium was renewed twice. (A) The significant upregulation of BDNF mRNA expression levels in EPI-NCSCs. The expression level of GDNF also elevates significantly following this treatment compared with that in the control group. Comparison of mRNA expression levels for BDNF (B) and GDNF (C) after treatment with VPA and crocin, alone and in combination, in EPI-NCSCs for 7 days, during which the medium was renewed twice. Values are mean ± SEM (n=3), $p<0.001$***, $p<0.01$**, $p<0.05$*.

tenance, and restoration of neurons in the CNS. The lack of these factors has been associated with several neuropathological conditions, and treatment modalities using these factors represent robust therapeutic options for neurodegenerative disorders (Allen et al., 2013). Here, we evaluated the mRNA expression levels of BDNF and GDNF in EPI-NCSCs following 7 days of treatment with VPA, crocin, or the co-treatment of VPA and crocin, under conditions in which the whole medium was renewed on the third and fifth days. Neurotrophic factors, such as BDNF and GDNF, activate the Ras-Raf-MEK-ERK signaling pathway, which causes neuronal growth, regeneration, and neurogenesis (Allen et al., 2013). After neurotrophins bind to their receptors, they regulate and activate MAPK, PI3K, and phospholipase Cγ (PLC γ) (Chen et al., 2013). GDNF enhances neuronal survival in the CNS and peripheral nervous system and influences a large population of cells, including sensory and motor ganglia and Purkinje cells in the cerebellum. BDNF also plays a role in the maintenance of sensory and motor neurons in the spinal cord (Allen et al., 2013).

Neural restoration and the replacement of lost cells are two important goals in the treatment of neurodegenerative disorders. Other aims during the treatment of neurodegenerative disorders include the enhancement of axonal repair, increased growth factor expression, the neutralization of inhibitory factors, reductions in glial scar formation, and improved regeneration (Bunge 2008). Numerous studies have reported that EPI-NCSCs are capable of differentiating into both neuronal and glial cells (Sieber-Blum 2006; 2010; Sieber-Blum and Grim 2004). In 2010, Sieber-Blum claimed that EPI-NCSC transplantation into an animal model of SCI resulted in a series of effects, including the replacement of glial and neuronal cells, trophic factor support, the induction of angiogenesis, the formation and regulation of glial scars, and the reinforcement of repair mechanisms, which improved the recovery of SCI mice (Sieber-Blum 2010).

In contrast, VPA is currently being used in clinical trials to treat seizures, depression, and epilepsy (Chateauvieux et al., 2010). VPA exerts effects on several cell signaling pathways, via its HDAC inhibitory functions. HDAC inhibition causes the hyperacetylation of core histones, which leads to gene activation or the suppression and regulation of gene expression. Therefore, VPA, *via* HDAC inhibition, can increase the acetylation of histones, leading to the gene expression of neurotrophic factors (Wu et al., 2008), which play important roles in neuroprotection and neurogenesis, by protecting surviving neurons from death and stimulating the growth of neural cells (Chu et al., 2015). In an animal model of SCI and under conditions of cellular excito-

toxicity, VPA functions via HDAC inhibition, which increases cell viability, enhances BDNF expression, prevents the activation of NF-kB, and inhibits ER stress (Scheuing et al., 2014). However, there are some studies describing an actual decrease in the gene expression levels of BDNF in the brain after VPA administration (Umka et al., 2010; Buzgoova et al., 2019). It has been well-documented that VPA performs an important role in the treatment of CNS damage, via the induction of neuronal differentiation and the enhancement of neural growth (Kim et al., 2005). Here, we detected a significant increase in the BDNF mRNA expression levels in EPI-NCSCs following treatment with 1 mM VPA for 7 days, although no significant alteration was detected in the GDNF mRNA expression level. Although our recent investigation showed increased BDNF and GDNF mRNA expression levels in EPI-NCSCs following VPA treatment (Pandamooz et al., 2019), here, we revealed that the expression of both BDNF and GDNF mRNA were upregulated more strongly if the medium was renewed on the third and fifth days.

Additionally, numerous studies have examined the antioxidant, anti-inflammatory, anti-tumor, cytotoxicity, and neuroprotective effects of crocin (Vahdati Hassani et al., 2014; Wang et al., 2015; Samini and Samarghndian 2016). In recent years, there has been an increasing amount of literature describing the effects of crocin on neurodegenerative disorders, such as Alzheimer's disease, depression, anxiety, and schizophrenia. Crocin inhibits apoptosis through the PI3K/AKT signaling pathway and increases the Bcl-2/BAX ratio (Khazdair et al., 2015). Our result showed that crocin treatment can significantly increase the BDNF mRNA expression level. However, only a slight increase has been observed in the mRNA expression level of GDNF after crocin treatment compared with that of the control group, which was not statistically significant. A previous study reported that crocin increased CREB, BDNF, and VGF protein expression in the rat hippocampus (Razavi et al., 2017).

The most interesting finding was that the co-treatment of VPA and crocin significantly increased both BDNF and GDNF mRNA expression levels compared with treatment using VPA or crocin alone. These findings further support the concept of a novel combination therapy for EPI-NCSCs, using an HDAC inhibitor (such as VPA) and the natural product crocin, in *in vivo* neurodegenerative disorder models, to upregulate BDNF and GDNF, which are both essential for neural development and regeneration. We found that both VPA and crocin treatment significantly increased the levels of BDNF mRNA expression and that, moreover, VPA and crocin co-treatment caused an additive increase in the BDNF mRNA expression level.

Together, these results provide important insights into the co-treatment of VPA and crocin, which can upregulate BDNF and GDNF mRNA expression levels, revealing their synergistic effects.

Further research should be performed to investigate the co-treatment of EPI-NCSCs using VPA and crocin in *in vivo* models, by measuring neurotrophin protein levels, which may further support the invaluable combination of these strategies for the treatment of neurodegenerative disorders.

CONCLUSION

The present study was designed to determine the effects of VPA and crocin on the EPI-NCSC survival rate and proliferation and the expression of neurotrophic factors. One of the most significant findings that has emerged from this study is that the combination of VPA and crocin and the renewal of the culture medium every other day can affect the BDNF and GDNF mRNA expression levels in EPI-NCSCs.

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