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Selegiline pre-induction modulates valproic acid effects on NGF expression during neuronal-like differentiation of rat bone marrow mesenchymal stem cells in vitro

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Authors:
Alireza Abdanipour [1]
Erfan Teymoori [2]
Mohsen Alipour [2]
Hadi Feizi [2*]

1. Department of Anatomical Sciences, Faculty of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran.
2. Department of Physiology and Pharmacology, Faculty of Medicine, Zanjan University of Medical Sciences, Zanjan, Iran.

**** Corresponding author:***
Hadi Feizi
ORCID: 0000-0001-5115-2053
E-mail: hfeizik@zums.ac.ir

ABSTRACT

Introduction: Neurodegenerative diseases are characterized by progressive neuronal loss and synaptic dysfunction. Neurotrophic factors such as nerve growth factor (NGF) is key molecular components linked to neuronal survival and functional connectivity. Mesenchymal stem cells derived from bone marrow (BMSCs) are promising candidates for regenerative approaches due to their differentiation capacity and neurotrophic paracrine effects. Valproic acid (VPA), a histone deacetylase (HDAC) inhibitor, may epigenetically modulate gene expression and promote neurogenic programs. Selegiline, a selective MAO-B inhibitor with neuroprotective properties, may prime neurotrophic pathways.

Methods: Male rat bone marrow mesenchymal stem cells (rBMSCs) were expanded in culture. Cells were allocated to four groups (n=3 biological replicates/group): Control, Selegiline (20 µM, 24 h), VPA (6 µM, 48 h), and Selegiline pre-induction (20 µM, 24 h) followed by VPA (6 µM, 48 h). Neuronal-like morphology was qualitatively assessed by phase-contrast microscopy. Total RNA was extracted and RT-qPCR was performed using GAPDH as the reference gene. NGF relative expression was calculated using the 2^-ΔΔCt method.

Results: All treated groups exhibited neuronal-like morphological changes compared with fibroblast-like controls, with the most prominent neurite-like extensions and interconnected networks in the sequential Selegiline+VPA group. NGF expression showed a treatment-dependent pattern. Statistical analysis indicated a significant difference between VPA and Selegiline+VPA ($p<0.01$).

Conclusion: Selegiline pre-induction followed by VPA supports a more favorable neurotrophic profile in rBMSCs, reflected by increased NGF expression compared with VPA alone, alongside stronger neuronal-like morphology.

Keywords: BMSC; Selegiline; Valproic acid; NGF; Neuronal-like differentiation; RT-qPCR.

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Introduction

Neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), and Huntington's disease, share core pathological mechanisms such as progressive neuronal loss, synaptic impairment, protein aggregation, neuroinflammation, and reduced capacity for endogenous repair. Because mature neurons have limited regenerative potential, current clinical therapies are largely symptomatic rather than disease-modifying, motivating the development of regenerative and neuroprotective strategies(1, 2).

A central molecular theme in neurodegeneration is disruption of neurotrophic support and synaptic integrity. Neurotrophins such as nerve growth factor (NGF) contribute to neuronal survival, axonal growth, differentiation, and synaptic plasticity through activation of signaling cascades including ERK/MAPK and PI3K/Akt (3-6).

Mesenchymal stem cells (MSCs), particularly bone marrow-derived mesenchymal stem cells (BMSCs), have attracted considerable attention as a promising therapeutic approach for neurodegenerative diseases owing to their robust proliferative capacity, multilineage differentiation potential, including differentiation toward neuron-like phenotypes, and potent immunomodulatory properties. Beyond their differentiation capabilities, MSCs exert significant paracrine effects through the secretion of neurotrophic factors, cytokines, and extracellular vesicles, which contribute to neuroprotection, modulation of the inflammatory

microenvironment, and promotion of neural tissue repair and regeneration (7-9).

Pharmacological priming is one approach to modulate MSC fate and trophic profiles. Selegiline, a selective monoamine oxidase-B (MAO-B) inhibitor used clinically in Parkinson's disease, has been reported to exert neuroprotective and neurotrophic effects and to influence expression of neurotrophins in experimental models(10). Valproic acid (VPA), widely used as an antiepileptic and mood stabilizer, also functions as an HDAC inhibitor and can regulate gene expression through epigenetic mechanisms, with reported context-dependent roles in neural differentiation and survival (11, 12).

Neurodegenerative disorders are a major and growing global health burden. Given the importance of NGF and synaptic markers in neuronal viability and network functionality, and the promise of BMSCs as a regenerative platform, this study investigated whether VPA alters the expression of NGF in rBMSCs, and whether selegiline pre-induction modifies the response to VPA.

Materials and methods

Study design

An in vitro study was conducted using cultured rBMSCs. Four experimental groups were considered as; Control (DMEM + FBS), Selegiline (20 µM, 24 h), VPA (6 µM, 48 h), and Selegiline(20µM,24h) plus VPA(6µM,48h). Each group included n=3 biological replicates, and RT-qPCR reactions were run on only one biological sample.

rBMSC isolation and culture

Bone marrow mesenchymal cells were harvested from 28-day-old rats under deep anesthesia and cultured in 25 cm2-adherent flasks in Dulbecco's modified Eagle's low-glucose medium (DMEM; Sigma-Aldrich) with 10% fetal bovine serum (FBS; Gibco), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich). The isolated cells were incubated at 37°C in 5% CO2 for 2 days. Adherent cells were harvested and subcultured, and the culture medium was changed every 2 days until the cells were 70 - 80% confluent. Cells were harvested with trypsin-EDTA (0.25%; Sigma-Aldrich) and passaged 3 times (P3) (13).

Drug preparation and treatments

Selegiline was prepared and applied to achieve a final well concentration of **20 µM** for **24 h (14).** VPA was applied at **6 µM** for **48 h (15)**. For the sequential group, cells were first exposed to selegiline (24 h) followed by VPA (48 h) after medium replacement. During treatment periods, drug-containing solution was supplemented every 6 h as described in the protocol.

Morphological assessment

Cells were monitored under phase-contrast microscopy at defined time points. Neuronal-like changes were documented qualitatively based on:

- Shift from spindle/fibroblast-like morphology to neuron-like cell bodies,
- Neurite-like thin elongated processes,
- Reduced cytoplasmic spread and more prominent soma,
- Formation of interconnected cellular networks.

RNA extraction, cDNA synthesis, and RT-qPCR

Total RNA was extracted using column-based procedures, quantified by Nanodrop, and reverse-transcribed to cDNA. RT-qPCR was performed with SYBR Green chemistry using primers for NGF and GAPDH. Cycling conditions: 95°C 10 min; 40 cycles of 95°C 15 s and 60°C 60 s, followed by melt curve analysis. The sequences of primers used for RT-qPCR are listed in Table 1. Relative expression was calculated using 2^-ΔΔCt, with GAPDH as reference and control as calibrator. NTC and additional no-RT controls were included for quality control.

Table 1. Sequences of primers used for quantitative real-time PCR (RT-qPCR).

Primer	Sequence (5' → 3')	Amplicon length (bp)
GAPDH	Forward: TTGTCAGCAATGCATCCTGCAC Reverse: GTCTGGGATGGAATTGTGAG	158
NGF	Forward : TCATCCACCCACCCAGTCTTCC Reverse : TAATGTTCACCTCGCCCAGCAC	133

Statistical analysis

Statistical analyses were performed using SPSS software version 15 (IBM; Armonk, New York, United States). All data are presented as means ± Standard Error of Mean (SEM) from 3 independent experiments. The one-way ANOVA followed by Tukey's post hoc was used to make a statistical comparison between groups. The level of significance was set at $P < 0.05$.

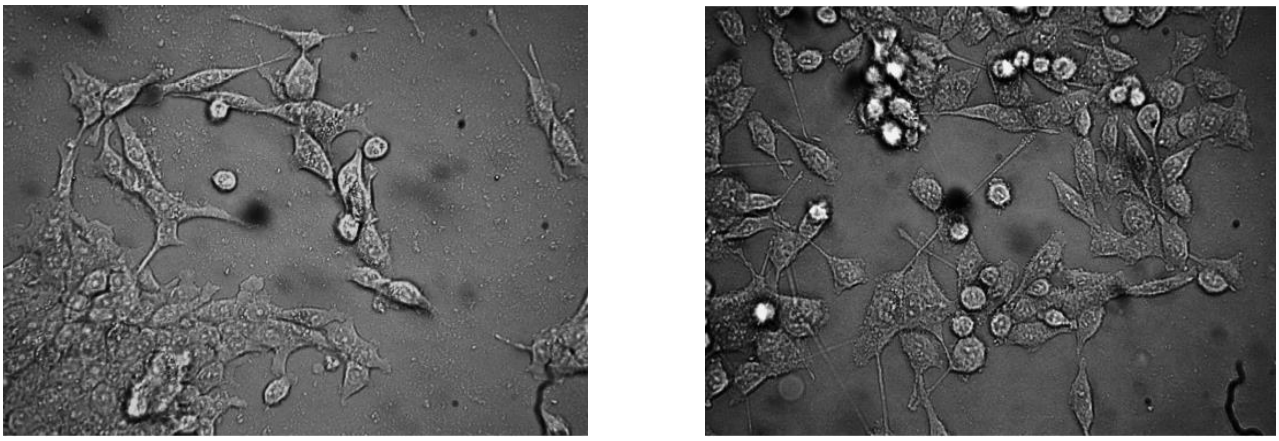
Results

Neuronal-like morphological changes

Control cells maintained typical MSC morphology (Figure 1). adherent, spindle-shaped, fibroblast-like). Selegiline-treated cells developed neurite-like processes and neuron-like shapes over time (Figure2). VPA-treated cells exhibited reduced cytoplasmic spread, more prominent soma, and short processes with network-like contacts (Figure 3). The sequential Selegiline+VPA group showed the most pronounced neuronal-like morphology with extensive processes and more evident intercellular network formation (Figure 4).

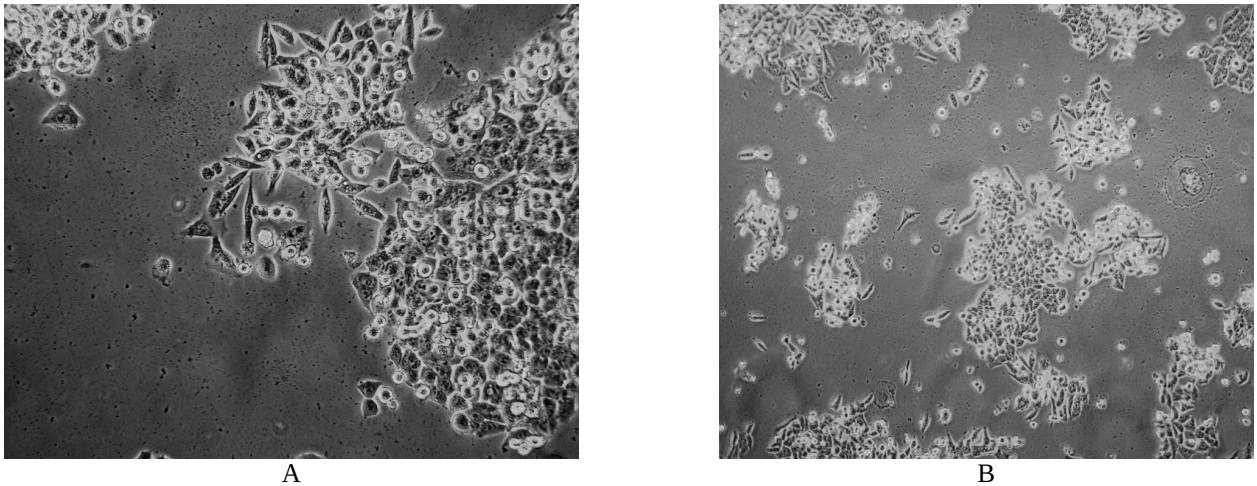
NGF expression (RT-qPCR)

Statistical comparison between groups was performed based on One-way ANOVA on ΔCt values and Tukey's post hoc test was used for pairwise comparisons. According to the results of Tukey's test, the difference in NGF expression between the VPA group and the selegiline + VPA group was significant.One-way ANOVA on ΔCt indicated significant group differences. Tukey post-hoc analysis identified a significant difference between **VPA vs Selegiline+VPA ($p<0.01$)**, indicating that selegiline pre-induction reverses or counteracts the NGF-lowering pattern observed with VPA alone. While the difference between other pairs of groups was not statistically significant ($p < 0.05$). Therefore, these data indicate that the combined treatment of selegiline with VPA can significantly increase NGF expression compared to VPA treatment alone and possibly suggest a modulatory or synergistic effect of selegiline in regulating NGF expression.



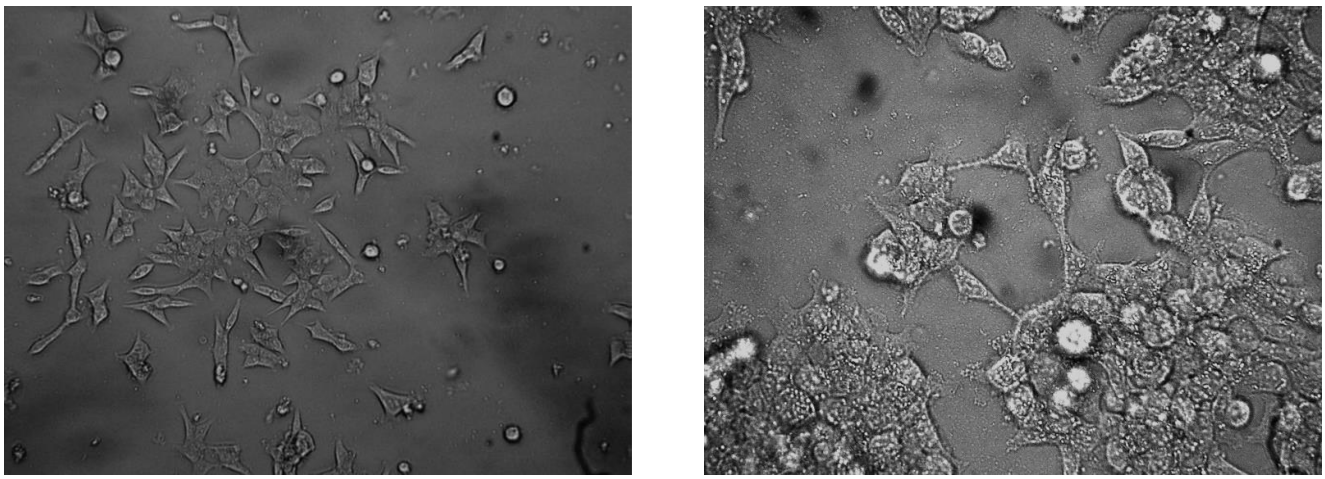
A B

Figure 1. Control cells morphology. The cells in the control group are spindle-shaped and fibroblast-like.



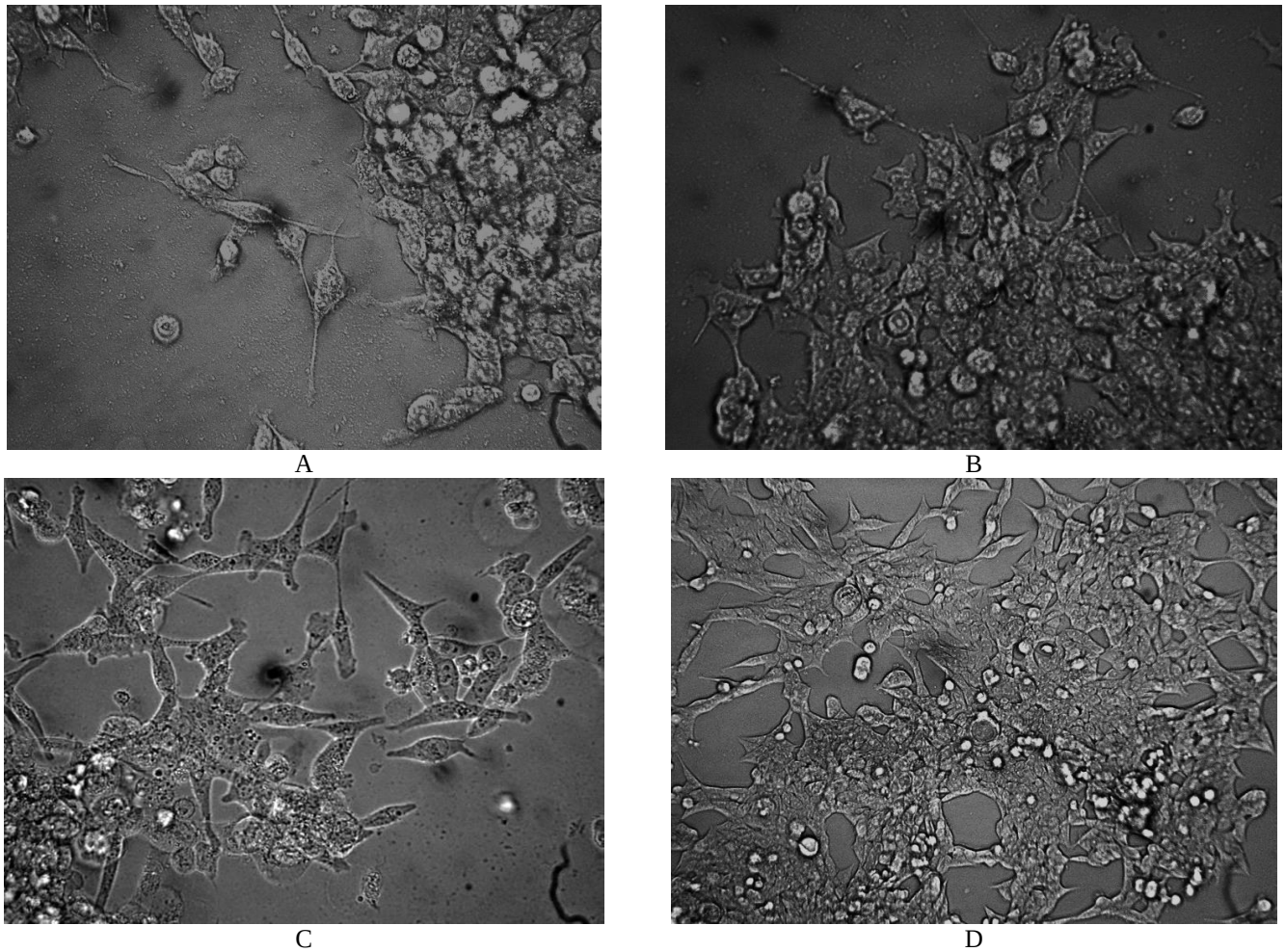
A B

Figure2. Selegiline-treated cells. In this group, a change in cell shape from a spindle shaped and fibroblast-like state to neuron-like cells was observed, as well as the appearance of short and long processes such as dendrites and axons. (A: 12 hours after induction with selegiline – B : 18 hours after induction with selegiline.)



A B

Figure 3. VPA-treated cells. In this group, a decrease in cytoplasmic width, a more prominent cell body, and the formation of networks of interconnected cells with short cell processes were observed and recorded.



A B C D

Figure 4. The sequential Selegiline+VPA group . In this group, cell shape changes from a spindle-shaped and fibroblast-like state to neuron-like cells, the appearance of short and long processes such as dendrites and axons, and the formation of networks of interconnected cells with cell processes were observed.(A,B : 24 hours pre-induction with selegiline + 12 hours of induction with valproic acid – C,D: 24 hours pre-induction with selegiline + 24 hours of induction with valproic acid.)

Discussion

Morphological Evaluation of Neuron-Like Differentiation

In the control group, cells maintained the typical mesenchymal morphology characterized by spindle-shaped, fibroblast-like appearances. These observations are consistent with previous reports demonstrating that primary rBMSCs exhibit an adherent spindle/fusiform morphology and may develop whirlpool-like growth patterns at higher cell densities (16).

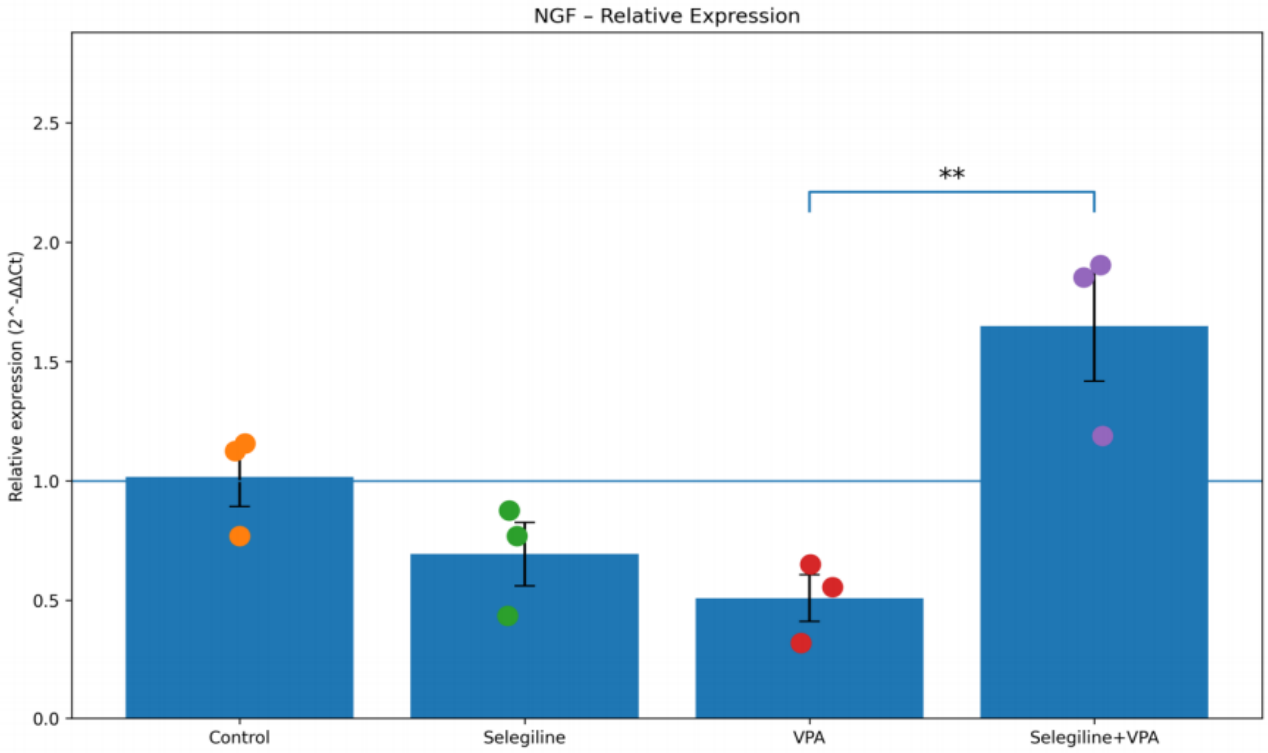


Figure 5. Relative expression of the NGF. In this graph, the bars represent the mean relative expression in each group and the error bars represent the standard deviation of the data as Mean ± SEM. The colored dots on each column also represent the values of each biological replicate (n=3). ($p < 0.01$; marked with ** in the graph).

In the selegiline-treated group, marked morphological alterations toward a neuron-like phenotype were observed, including the formation of short and elongated cellular processes resembling dendrites and axons. These findings are in agreement with the well-established neuroprotective and neurotrophic properties of selegiline and suggest that this compound promotes a neuronal predisposition in mesenchymal stem cells, although functional neuronal maturation was not assessed in the present study. Similar observations were reported by Kashani et al who demonstrated that selegiline induces neuron-like differentiation of bone marrow-derived mesenchymal stem cells and promotes the development of neurite-like extensions (17).

Treatment with VPA also induced substantial morphological changes, including reduced cytoplasmic volume, a more prominent cell body, and the formation of interconnected cellular networks with short neurite-like processes. These alterations may reflect cytoskeletal remodeling and changes in cell–cell interactions associated with neural differentiation. Consistent with our observations, Jeong et al reported that VPA, acting as a histone deacetylase (HDAC) inhibitor, enhances neural precursor-associated pathways and facilitates the acquisition of neuron-like morphological characteristics, including increased neurite length and number (18).

Interestingly, the combined treatment group (selegiline+VPA) exhibited the most pronounced neuron-like morphology, characterized by extensive neurite outgrowth and the formation of well-defined cellular networks. These findings suggest a possible priming effect of selegiline, whereby pre-induction with selegiline enhances the responsiveness of rBMSCs to subsequent VPA treatment and promotes the acquisition of neuronal characteristics. Similar synergistic effects have been reported by Abdanipour et al. who demonstrated that combined treatment strategies resulted in increased neurite formation and larger soma size compared with individual treatments (14).

Effects of Selegiline and VPA on NGF Gene Expression

Analysis of relative gene expression revealed distinct effects of the different induction protocols on **NGF** expression. Following normalization to GAPDH and calculation using the 2^−ΔΔCt method, NGF expression was approximately 0.89-fold in the selegiline group and 0.50-fold in the VPA group relative to the control. In contrast, the combined treatment

group exhibited a relative expression level of approximately 1.63-fold, indicating an increase above baseline levels. Statistical analysis using Tukey's post hoc test further demonstrated that NGF expression in the VPA group was significantly lower than that observed in the combined treatment group.

The biological interpretation of these findings may be related to the complex and context-dependent actions of VPA on gene regulation. As an HDAC inhibitor, VPA exerts broad epigenetic effects that can either activate or repress gene expression depending on treatment duration, concentration, cell type, and differentiation status. Under the experimental conditions employed in the present study, VPA alone did not effectively activate NGF-associated pathways and instead was associated with a reduction in NGF expression.

Conversely, the inclusion of selegiline in the induction protocol appeared to shift the regulatory balance toward enhanced NGF expression. This effect may be mediated through several mechanisms, including attenuation of oxidative stress, neuroprotective activity, modulation of intracellular signaling pathways, and enhancement of neurotrophic support. Therefore, the present findings suggest that selegiline may counteract or mitigate potential suppressive effects of VPA on NGF expression.

Previous studies support the notion that the effects of VPA on neural differentiation and neurotrophic signaling are highly dependent on experimental context. Satoh et al (19). demonstrated that VPA could enhance neural characteristics and functional properties of adipose-derived stem cells under specific induction conditions, highlighting the importance of induction protocols in determining cellular outcomes. In contrast, Umka J et al. reported that subchronic VPA administration in rats reduced hippocampal BDNF levels and was associated with impaired cognitive performance, indicating that VPA may exert adverse effects on neurotrophic pathways under certain conditions (20).

The results obtained in the selegiline group are also consistent with previous evidence suggesting that the effects of selegiline on neurotrophic signaling may vary depending on cellular context. Gerlach et al. emphasized that selegiline does not invariably activate neurotrophic pathways in all experimental systems and that its biological effects may depend on factors such as MAO-B inhibition, monoamine metabolism, treatment duration, and culture conditions (21). Nevertheless, Kashani et al. reported that selegiline significantly increased the expression of neurotrophins, including NGF, in stromal stem cells, supporting its role in enhancing neurotrophic capacity and neuronal support mechanisms (17).

Conclusions

In summary, the induction protocols employed in the present study exerted differential effects on neurotrophic characteristics in rBMSCs. Under the conditions examined, VPA treatment alone was associated with reduced NGF expression, whereas pre-induction with selegiline followed by VPA treatment improved the overall gene expression profile. Specifically, NGF expression was significantly elevated in the combined treatment group compared with the VPA-only group. These findings suggest that selegiline may alleviate potential adverse effects of VPA on neurotrophic signaling pathways and promote a more favorable molecular environment for neuronal differentiation. Collectively, the results support the potential utility of selegiline as a priming agent to enhance the neurogenic and neurotrophic responses of mesenchymal stem cells subjected to VPA-based induction protocols.

Author′s contribution

A. Abdanipour contributed to the interpretation and observations, E. Teymoori and M. Alipour data collecting and analysis. The principal investigator, H. Feizi, acquired the funding to support the project, oversaw the project progress, and wrote the paper.

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Data availability

Data available on request from the authors.

Ethics approval

All experimental procedures were performed in accordance with Zanjan University of Medical Sciences guidelines for the care and use of laboratory animals (Ethical approval code:

IR.ZUMS.BLC.1404.050).

Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Artificial intelligence (AI)-assisted technology: ChatGPT was used for language editing.

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