Original Article

Directing Rat Hair Follicle Stem Cells Toward Neuronal Lineage With Enhanced Trophic Factor Expression

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Abstract

Background: Hair follicle stem cells (HFSCs) are promising candidates for cell-based therapies in neurodegenerative diseases because of their ability to differentiate into neural lineages and exert paracrine effects in damaged tissues. However, their clinical application faces challenges, particularly in efficiently guiding them toward neural lineages. This study explores using chick embryo extract (CEE) to enhance HFSCs' secretory capacity and neuronal differentiation.

Materials and Methods: HFSCs from rat whisker pads were cultured in growth medium supplemented with either 20% FBS or a combination of 10% FBS and 10% CEE, transitioning to 20% FBS after the first subculture. We conducted gene expression profiling of lineage commitment markers and neurotrophic factors in both experimental groups, alongside morphological assessments and protein expression analyses.

Results: CEE supplementation during migration increased neuronal differentiation, evidenced by more cells with neurites and higher MAP2 expression at both the gene and protein levels. CEE also inhibited the expression of PDGFR-α, indicating a suppression of differentiation toward Schwann cells. Furthermore, we observed increased levels of trophic factors such as BDNF and VEGF at passage 3 induced by CEE supplementation.

Conclusions: Enhancing the neuronal lineage commitment of hair follicle stem cells (HFSCs) and boosting the expression of trophic and angiogenic factors through short-term CEE preconditioning during their migratory stage presents a compelling approach. This strategy holds great promise in enhancing the effectiveness of stem cell-based therapies for neurological disorders.

Keywords: Hair follicle, neuronal differentiation, preconditioning, stem cells, trophic factors

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Introduction

Central nervous system (CNS) diseases encompass a spectrum of debilitating conditions marked by malfunctioning neurons and glial cells. Over the last decades, cell-based therapy has emerged as an effective therapeutic strategy for combating CNS disorders, both in fundamental research and clinical applications.[1] Stem cells, in particular, have captured the spotlight because of their unique potential to directly differentiate into neural cells, astrocytes, and oligodendrocytes. Moreover, they release a diverse range of neurotrophic factors into damaged tissue. This dual capability holds tremendous promise for CNS regeneration.[2] Among stem cells from diverse sources, hair follicle stem cells (HFSCs) stand out as an appealing candidates. These stem cells are remnants of the transient embryonic neural crest that persist in the bulge region of hair follicles through adulthood.[3,4] HFSCs offer many advantages for cell-based therapies, including

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remarkable plasticity, ease of isolation, ready accessibility, high purity, and the distinct ability to undergo autologous transplantation without fear of graft rejection.[5,6] In addition to the direct differentiation of HFSCS into multiple neural lineages, they also express a variety of neurotrophic and growth factors. These factors, significantly contribute to the process of CNS regeneration after transplantation, making HFSCs an attractive option for therapeutic interventions.[7] However, the full potential of stem cells in repairing injured CNS tissues faces significant hurdles, primarily stemming from insufficient differentiation.[8] Over recent years, preconditioning with various physical, chemical, and biological stimuli has emerged as a pivotal technique. It enhances stem cell functionality in laboratory settings and boosts the effectiveness of HFSC transplantation in tissue engineering and regenerative medicine.[9-12] Within this complex landscape, growth factors and neuropoietic cytokines play a prominent role in steering the cell fate decisions of neural crest cells.[13] One intriguing substance, chick embryo extract (CEE), is derived from whole chick embryos and has found application in the *in vitro* culture of neural crest cells, neuronal cells, neuroepithelial cells, embryonic stem cells, and notably HFSCS.[14-16] Noteworthy prior investigations have reported accelerated growth rates in neural crest cells cultivated in the presence of CEE[17,18] However, the influence of CEE on the differentiation potential of stem cells remains largely unexplored. In a recent breakthrough, our research group has uncovered that the long-term preconditioning of HFSCs with CEE, initiated during their early migration stages and extending through later stages, can effectively steer their fate toward Schwann cells. Intriguingly, proteomics analysis of CEE content has uncovered the presence of essential components for neuronal differentiation as well.[19] Previous studies have demonstrated that environmental signals encountered during the migratory stage are critical in directing neural crest stem cells to differentiate into different lineages upon reaching their destination.[20,21] In light of these groundbreaking findings, this study takes a pioneering step by incorporating CEE during the initial stages of HFSC culture. This manuscript aims to explore the effects of short-term CEE preconditioning on the fate of HFSCs, particularly focusing on their differentiation toward neural lineages. In addition, we aimed to assess the transcript levels of genes crucial in neural lineage commitment. Moreover, our investigation involved the examination of four key trophic factors within HFSCs to determine whether this preconditioning regimen can influence their differentiation toward neural lineages and importantly, enhance the expression of trophic factors. Ultimately, our aim is to improve the potential therapeutic benefits of preconditioned HFSCs for prospective transplantation in diverse neurological conditions.

Materials and Methods

Animal care and protocols adhered strictly to the guidelines approved by the Animal Care Committee of Shiraz University of Medical Sciences, in accordance with internationally recognized ARRIVE guidelines. Furthermore, this study received approval from the Research Ethics Committees of Laboratory Animals at Shiraz University of Medical Sciences, with the approval ID: IR.SUMS.AEC.1401.086.

Preparation of chick embryo extract

The process of preparing chick embryo extract is performed with the careful harvesting of 11-days-old chick embryos, which are delicately collected into pre-chilled tubes. After a triple wash with ice-cold phosphate-buffered saline (PBS), the embryos underwent further processing. The heads were carefully excised, leaving the bodies which were then homogenized before centrifugation at 12,000 g, 4°C for 30 minutes.[14] Subsequently, the resulting supernatant was filtered through 0.45 and 0.22-μm filters, and samples were preserved at -80°C until required. Importantly, the protein composition of the chick embryo extract had been previously identified in our work.[19]

Isolation and in vitro expansion of HFSCS

The HFSCS were isolated from the bulge region of sizable hair follicles located in the rat whisker pad, after established procedures.[22] Initially, the hair follicle was dissected, and the bulge area was cut and explanted onto pre-coated plates, as previously detailed.[23] In this study, approximately 40 bulges were dissected from each animal and assigned to either the FBS group or the CEE-FBS group. To ensure triplicate samples, three animals were used for each experimental group in this process. The explanted bulges were grown in the presence of alpha-modified minimum essential medium (α-MEM, ShellMax, # M4140) supplemented with 1% L-Glu (ShellMax, #GB510) and 1% penicillin/streptomycin (ShellMax, # P3790).[24,25] Depending on the experimental group, cultures were further supplemented with either 20% FBS (Bio Idea, #BI1201) (Experimental Group 1, i) or a combination of 10% FBS and 10% CEE (Experimental Group 2, ii). Figure 1a depicts the experimental groups and illustrates the type of medium supplementation used for each group (green lines). On day 13 of the experiment, the migrated stem cells underwent subculturing using 0.25% Trypsin/EDTA solution (ASAgene, # LM-T17020). This process involved carefully detaching the cells from the culture surface, enabling their subsequent propagation and expansion in a fresh growth medium. Importantly, after the first subculture, stem cells in the second experimental group (10% FBS plus 10% CEE) were supplemented with 20% FBS. Figure 1a provides a schematic representation of the study protocol, illustrating the duration of HFSC culture and delineating the precise timing of each experimental procedure.

Immunofluorescent staining

The immunofluorescent staining procedure was performed following a well-established protocol[26] to verify the identity of migrated stem cells at passage 1 and to evaluate their differentiation potential toward neuronal or glial lineage at passage 3. In this experiment, cells were seeded at a density of 7.5×10^4 cells in a 4-well plate and cultured for nearly

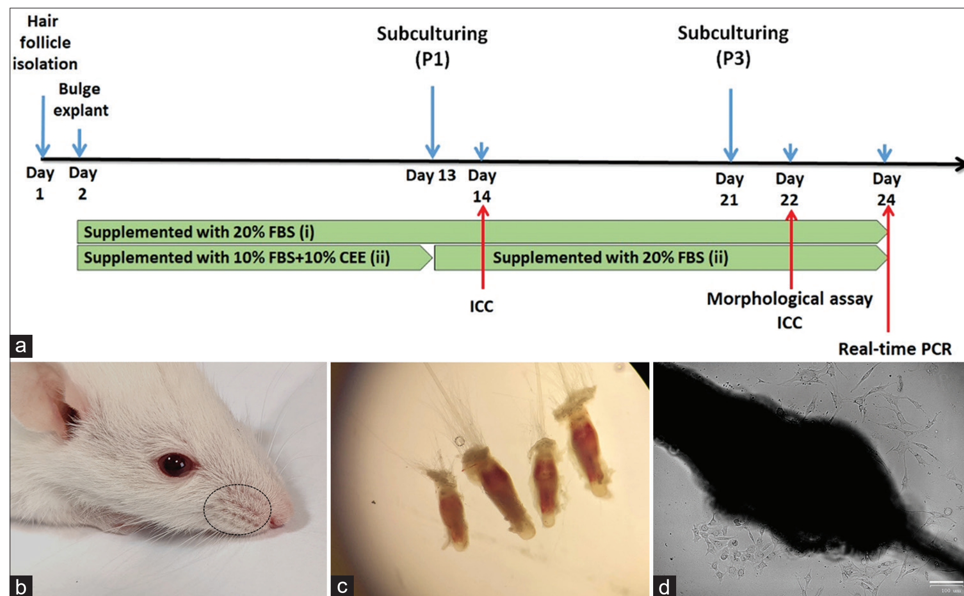

Figure 1: Isolation of rat hair follicle stem cells. Schematic timeline protocol of study (a). The whisker pad of a rat (b) was cut and large hair follicles were isolated (c). The bulge area of the hair follicle was carefully microdissected and explanted. Few days later, migrated stem cells were observed around the bulge (d), scale bar: 100 μm

24 hours. After this incubation period, the cells were fixed with 4% paraformaldehyde (PFA) for 15 min. After three washes with PBS-T and subsequent 30-minute blocking step (0.1% Triton X-100, 1% FBS, 10% normal goat serum prepared in PBS), the cells were incubated with primary antibodies [listed in Table 1] overnight at 4°C. To ensure the specificity of the immunostaining, each marker was addressed individually to prevent cross-reaction of antibodies.

On the subsequent day, the cells were subjected to a triple wash before being reblocked with 3% bovine serum albumin for 10 minutes. After this, they were incubated with secondary fluorescent antibodies as follows: goat anti-mouse IgG AlexaFluor488 (1:1000, Abcam, #ab150117), and goat anti-rabbit IgG AlexaFluor488 (1:1000, Abcam, #ab150085) for 2 h at room temperature. Finally, cell nuclei were counterstained with DAPI, and images were captured using a ZOE fluorescent microscope (Bio-Rad, USA).

Table 1: The list of primary antibodies used in immunostaining experiment with their respective concentrations

Primary antibody	Company	Cat. no.	Con.
Mouse anti-nestin monoclonal antibody	Abcam	ab6142	1:50
Rabbit anti-SOX10 polyclonal antibody	Proteintech	10422-1-AP	1:100
Mouse anti-beta III tubulin monoclonal antibody	Abcam	ab78078	1:1000
Mouse anti-MAP2 monoclonal antibody	Abcam	ab11267	1:200
Rabbit anti-GFAP monoclonal antibody	Abcam	ab33922	1:200
Rabbit anti-PDGFRα polyclonal antibody	Abcam	ab61219	1:200

Morphological assessment

To assess cellular morphology and quantify neurite formation, cells from both experimental groups were seeded onto a 12-well plate at a density of 1.5×10^5 cells per plate and incubated in their respective growth medium overnight. This density was carefully chosen to achieve a balance, as excessively high cell numbers can lead to cellular aggregation, rendering accurate neurite length measurements unfeasible.[26] The following day, the cells were fixed with 4% PFA for 15 minutes. After fixation, cells were rinsed with PBS and stained with 0.5% crystal violet solution. To quantify neurite formation, 33 randomly selected images from each group were analyzed, and the percentage of cells exhibiting neurites within each image was determined. Neurite length was quantified by manually tracing the longest neurite per cell (using ImageJ Fiji version 1,52r software, NIH, USA). Analysis was conducted for all cells in a field exhibiting identifiable neurites and where the entire neurite arbor could be visualized. The length of each neurite was measured from the edge of the nucleus to its apical end. Only neurites devoid of contact with neighboring cells were included in the evaluation. To ensure accuracy and reliability, at least two team members independently assessed neurite length in a blinded fashion. Cellular area was evaluated using the measuring tool in ImageJ software, allowing for accurate quantification of the cell area within the experimental samples.

RNA extraction and quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from both experimental groups at passages 1 and 3 to assess the expression of neurotrophic and angiogenic factors at both stages, whereas cellular markers

were evaluated at passage 3. The procedure was conducted according to the manufacturer's guidelines (Cat. No: YT9063; Yekta Tajhiz Azma, Iran). After DNase I treatment to eliminate genomic DNA contamination, 1-µg of total RNA was used for cDNA synthesis using a reverse transcriptase kit (Cat No: YT4500; Yekta Tajhiz Azma). Subsequently, quantitative real-time PCR was performed in triplicate on an ABI StepOne Real-Time PCR system. This involved using specific primers [listed in Table 2] and RealQ Plus 2x Master Mix Green (Cat. No: A325402; Ampliqon, Denmark) with the following thermal cycling conditions: initial denaturation at 95.0°C for 15 min, followed by 40 cycles of denaturation at 95.0°C for 20 s and annealing/extension at 60.0°C for 1 min. The Hprt gene was used as the housekeeping gene, and the $2^{-\Delta\Delta Ct}$ method was used to determine the relative expression changes in the selected genes.[27] This comprehensive approach ensured accurate quantification and analysis of gene expression levels within the experimental samples.

Statistical analysis

The Statistical analysis was performed using GraphPad Prism (Version 7.03, GraphPad Software Inc., San Diego, CA). The data are presented as mean ± SEM. An independent *t*-test was conducted to determine the statistical differences among the groups, preceded by assessing the normality of all data using the Shapiro-Wilk test. Here, $P < 0.05$ was considered to be statistically significant.

Results

Migration dynamics and Nestin and SOX-10 expression of HFSCs at passage 1

Two days after bulge explantation, migrated stem cells were observed around hair bulges [Figure 1b-d]. After the first subculture of migrated stem cells in both experimental groups, we assessed the protein levels of Nestin and SOX-10. Nestin, a well-established marker of neuronal progenitor cells, is a commonly used marker for neural stem cells. It is also expressed in certain types of epithelial stem cells, including those found in hair follicles. SOX-10, on the other hand, is a transcription factor that plays a critical role in the development of neural crest-derived cells [Figure 2]. These immunostaining procedures align with established practices documented in previous studies that characterized and confirmed the identity of migratory HFSCs.[28,29] Our findings revealed robust expression of Nestin and SOX10 proteins in both experimental groups, validating the identity of migrated stem cells as HFSCs. This finding underscores the potential of these cells for neuronal and neural crest differentiation.

Preconditioning effects on the morphology of HFSCs at passage 3

The assessment of stem cell morphology at passage 3 [Figure 3a] revealed notable differences between the two experimental groups. In the FBS group, 83.87 ± 2.5% of cells exhibited neurites, a percentage significantly lower than that of the CEE-FBS group, where 96.19 ± 1.17% of cells displayed neurites [Figure 3b, ***$P < 0.001$]. Although there was variation in the percentage of cells with neurites, no significant difference in neurite length was observed between the groups (91.44 ± 1.68-µm vs. 89.61 ± 1.60-µm, $P = 0.43$, graph not shown).

Moreover, in the FBS group, 13.04 ± 1.75% of cells possessed neurites longer than 100-µm, whereas CEE preconditioning increased this proportion to 20.07 ± 2.12% [Figure 3c, *$P < 0.05$]. Collectively, these findings suggest that CEE preconditioning during the early stage of *in vitro* culture prompts a neural-like morphology in HFSCs. Despite the observation of longer neurites in the CEE-FBS group, no significant difference in cell area between the two groups was detected [Figure 3d, $P = 0.54$].

mRNA and protein expression analysis of HFSCs at passage 3

To investigate the potential of short-term CEE preconditioning during early *in vitro* culture on the fate of HFSCs, we assessed the expression of two well-known glial markers and two key neuronal markers at both mRNA [Figure 4a] and protein [Figure 4b] levels at passage 3. Specifically, we examined the transcription levels of GFAP and PDGFR-α, established glial markers. Although the expression of GFAP remained unchanged between experimental groups ($P = 0.50$), the transcript level of PDGFR-α was significantly reduced in the CEE-FBS group compared with the FBS group (**$P < 0.01$) [Figure 4a]. In addition, we evaluated neuronal markers, including βIII tubulin and MAP2, to

Table 2: List of primers

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon length [bp]
B-III tubulin	GCTGGAACGCATCAGTGTCTAC	GCACCACTCTGACCGAAGATAAAG	162
gfap	GGGACAATCTCACACAGGACCTC	CCTCCAGCGACTCAACCTTCC	162
Pdgfr-α	AACCGAGGAGAACAACAGTAGCC	AAGAATCCGTCATGCCGAGAGG	194
Map2	AAGCGGAAAACCACAGCAACAAG	TTCTCCTCCCTGTCTCCTGATACG	176
Bdnf	CGATTAGGTGGCTTCATAGGAGAC	CAGAACAGAACAGAACAGAACAGG	182
Gdnf	GCTGACCAGTGACTCCAATATGC	CCTCTGCGACCTTTCCCTCTG	192
Vegf	ACTTGAGTTGGGAGGAGGATGTC	GGATGGGTTTGTCGTGTTTCTGG	183
Ngf	CCCAATAAAGGCTTTGCCAAGGAC	GAACAACATGGACATTACGCTATGC	78
hprt	CCAGCGTCGTGATTAGTGATGATG	GAGCAAGTCTTTCAGTCCTGTCC	135

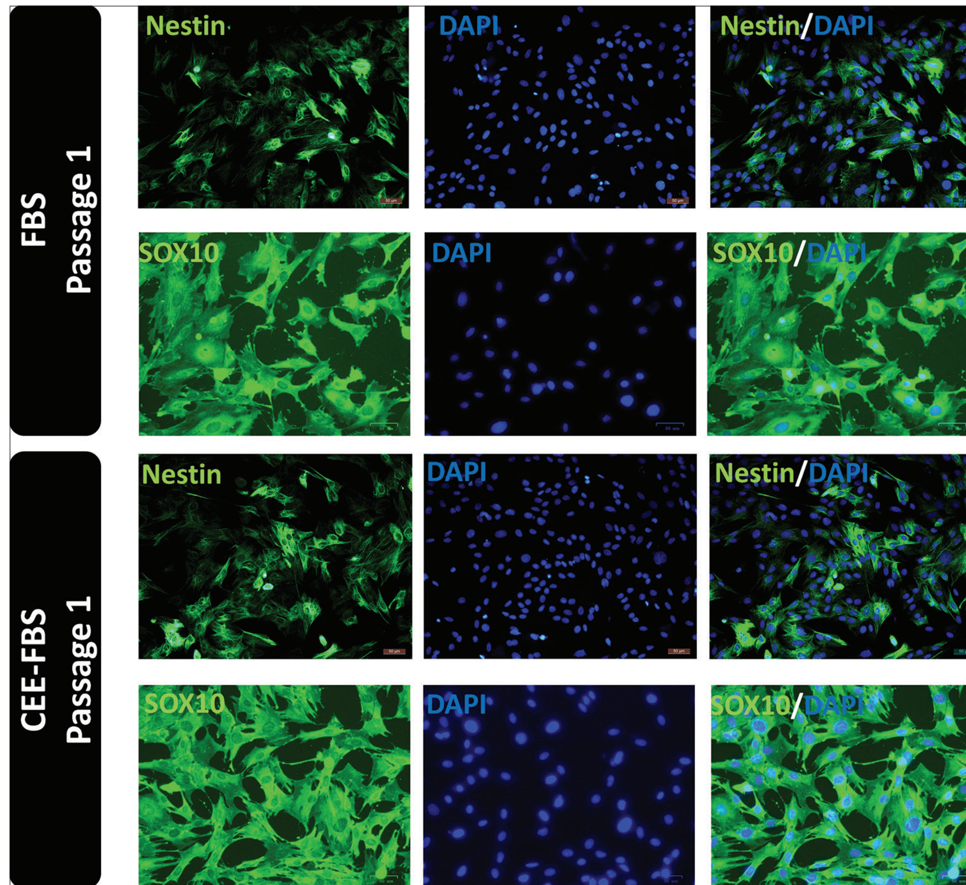

Figure 2: Nestin and SOX10 expression in hair follicle stem cells at passage 1. Immunofluorescence analysis demonstrates robust expression of nestin and SOX10 proteins in both experimental groups at passage 1. Cell nuclei were counterstained with DAPI, scale bar: 50-μm. The images provided represent examples of three distinct assessments for each immunostaining (n = 3)

explore the potential differentiation toward the neuronal fate. Although there was an observed increase in the expression of βIII tubulin in the CEE-FBS group, statistical analysis revealed that this increase was insignificant ($P = 0.49$). However, notably, the mRNA level of MAP2 showed a significant elevation in the CEE-FBS group compared with the FBS group (**$P < 0.01$). This finding suggests a clear inclination toward differentiation into the neuronal lineage [Figure 4a]. Analysis of protein expression patterns yielded same results. Immunostaining against same neuronal and glial markers revealed elevated expression of βIII tubulin and MAP2 in CEE-preconditioned stem cells [Figure 4b]. The increased expression observed in both mRNA and protein levels suggests that CEE-preconditioning during the migratory stage can effectively steer the fate of HFSCs toward the neuronal lineage.

Enhancing trophic factor expression in HFSCs through preconditioning

In addition to assessing cellular markers, we investigated the relative expression of key neurotrophic factors and an angiogenic factor at passages 1 and 3 in both experimental groups [Figure 5]. It is worth highlighting that HFSCs, also known as epidermal neural crest stem cells, possess the remarkable ability to express a variety of trophic factors.[30] This expression can undergo modulation under different treatment and culture conditions, as evidenced by several studies.[31,32] Initially, at passage 1, the expression levels of brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), nerve growth factor (NGF), and vascular endothelial growth factor (VEGF) exhibited no significant differences between the two groups. However, by passage 3, notable changes were observed. The transcript levels of BDNF and VEGF showed a significant increase in the CEE-FBS group (**$P < 0.01$ and *$P < 0.05$, respectively), whereas the expression of NGF decreased by approximately 50% compared with the FBS group (**$P < 0.01$). Conversely, the difference in GDNF mRNA expression between the groups was not statistically significant ($P = 0.17$) [Figure 5].

Discussion

Neural crest-derived stem cells are distributed throughout various tissues in adult organisms, constituting a significant reservoir of multipotent and autologous stem cells.[33] These cells have been harnessed in cell-based therapies for conditions such as spinal cord injury,[22,34] sciatic nerve injury,[35,28] and stroke.[36,37] Although the broad potential and accessibility of neural crest stem cells hold promise, there are

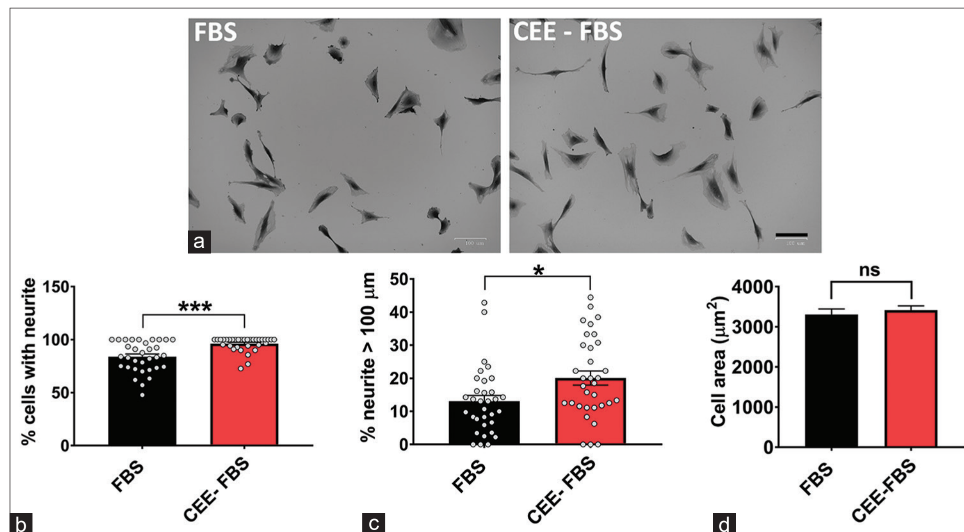

Figure 3: Morphological assessment of hair follicle stem cells. Representative images depicting the morphology of HFSCs from each experimental group (n = 33), with a scale bar of 100 µm (a). Statistical analysis demonstrates a significantly higher percentage of cells with neurites in the CEE-FBS group compared with the FBS group (b). Moreover, a greater proportion of cells in the CEE-FBS group exhibit neurite lengths exceeding 100-µm (c). Evaluation of cell area reveals no significant difference between the two groups (d). Data are presented as mean ± SEM. Statistical differences between groups were determined using *t*-test. $^{*}P < 0.05$ and $^{***}P < 0.001$ compared with the FBS group

notable challenges associated with their *ex vivo* expansion. These include the need to isolate sufficient quantities of stem cells and refine protocols for neuronal differentiation, highlighting the necessity for further advancements in culture conditions.

Previously, it has been demonstrated that environmental cues encountered during the migratory phase of NCSCs play a crucial role in dictating their cellular fate, necessitating the provision of a growth factor-enriched medium for optimal NCSC expansion.[38,20] Traditionally, NCSCs have been cultivated *in vitro* using FBS alongside selected growth factors.[39,40] However, the use of synthetic growth factors is constrained by their high cost, short half-life, and limited stability. Emerging evidence underscores the advantages of supplementing culture media with CEE during the *in vitro* propagation of HFSCs, as this complex mixture of growth factors surpasses the efficacy of individual synthetic growth factors.[14] Nonetheless, the impact of CEE supplementation on HFSCs during the migratory stage remains unexplored. In this study, we observed that migrated stem cells in both experimental groups displayed characteristic stellate morphology [Figure 1d] and expressed neural crest stem cell markers, confirming their identity as HFSCs [Figure 2]. Although the precise composition of growth factors and cytokines within CEE remains to be fully characterized, Ma *et al*. identified several key factors, including stem cell factor, NGF, EGF, IL-4, and IL-2[41] which likely contributes to the observed enhancement in HFSCs development in our study. Furthermore, recent investigations conducted by our research group using tandem mass spectrometry identified over 833 protein groups in CEE. These findings unveiled a spectrum of CEE proteins potentially involved in various cellular responses, including stem cell maintenance, migration, and differentiation.[19]

Our findings reveal that HFSCs supplemented with 10% FBS + 10% CEE during the migratory stage exhibit distinctive neural morphological features characterized by extended and longer neurites. Supporting this observation, Christman *et al*.[42] demonstrated that low concentrations of CEE can enhance the morphology and growth rate of the chicken embryo fibroblast cell line. This neurite outgrowth is a critical aspect of neural development and regeneration. It facilitates enhanced connectivity among neurons, enabling the establishment of connections and ultimately the formation of neural networks. These networks are vital for various functions, including information processing, memory formation, and sensory perception. Also, neurites serve as conduits for transmitting electrical signals and chemical cues between neurons and other cells within the nervous system. The extension of neurites promotes efficient signal transmission and integration, enabling coordinated responses to stimuli[43] The ability of HFSCs to promote neurite outgrowth represents a valuable strategy for harnessing the regenerative potential of stem cells and advancing treatments for various neurological conditions such as spinal cord injury, stroke, and neurodegenerative diseases[44-47] By fostering neurite outgrowth, HFSC-based therapies have the potential to facilitate neural repair and functional recovery in affected individuals.

Subsequently, we investigated the effects of CEE supplementation during the early culture period on the differentiation potential of these cells into neural lineages. Evaluation of neural lineage-specific genes revealed a significant increase in the expression level of MAP2, a neuronal marker, in the CEE-FBS experimental group compared with the FBS group. These findings suggest that short-term CEE preconditioning may promote the fate of HFSCs toward neuronal lineage while inhibiting their differentiation into the glial lineage. This aligns

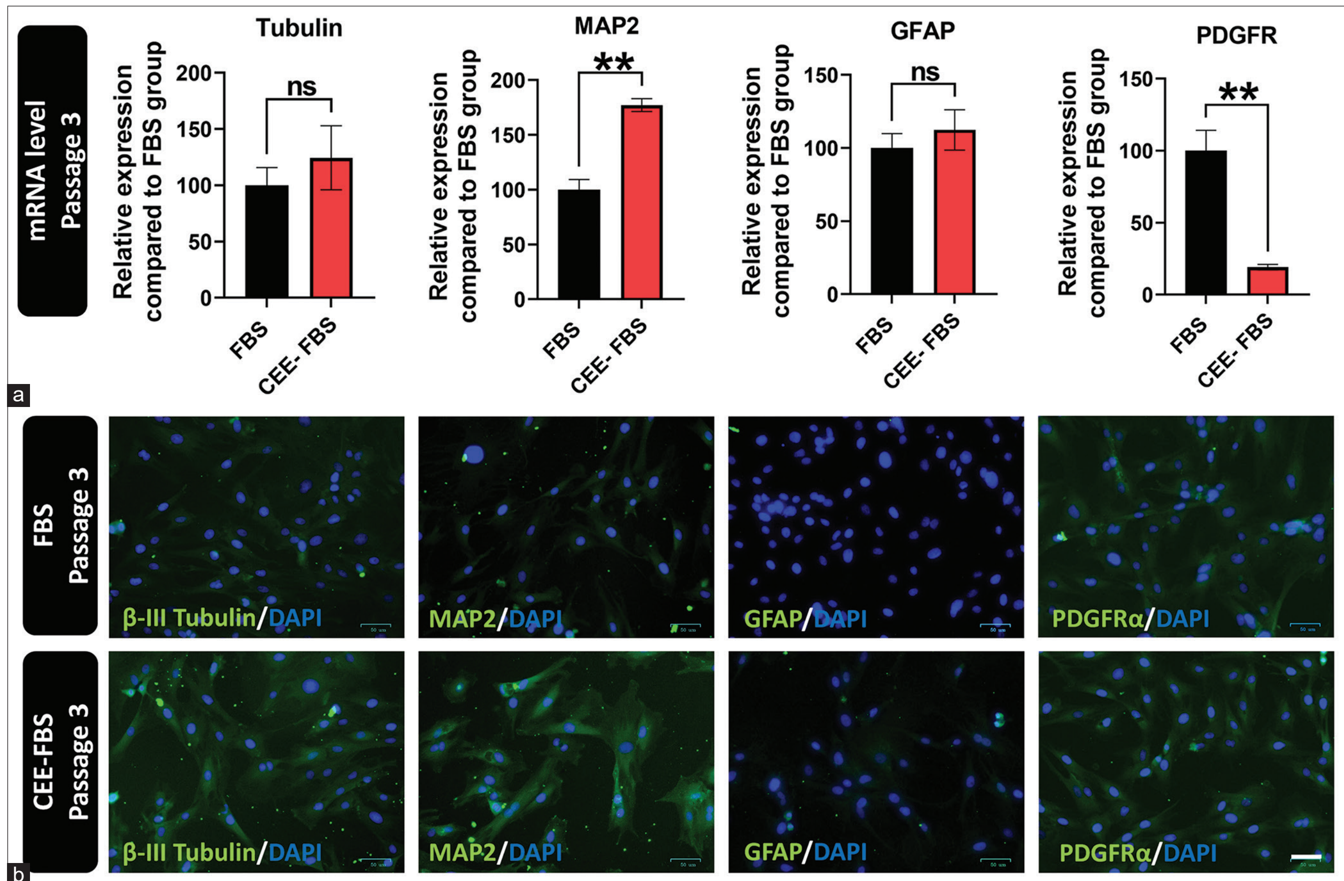

Figure 4: Analysis of mRNA and protein expression in hair follicle stem cells at passage 3. The mRNA expression level of MAP2 as a neuronal marker was significantly increased in the CEE-FBS experimental group. Although there was a rise in β-III tubulin expression within the CEE-FBS group, statistical analysis indicated that this increase was statistically insignificant. The expression of PDGFR-α as a glial marker was dramatically reduced, whereas no significant difference was detected in GFAP expression between groups (a). Values are mean ± SEM of three independent experiments. The difference between groups were identified by *t*-test. $^{**}P < 0.01$ versus the FBS group. Immunostaining highlights abundant expression of β-III tubulin and MAP2 in CEE-FBS group (b). Cell nuclei were counterstained with DAPI, scale bar: 50 μm. Images are examples of three different assessments for each immunostaining (n = 3)

with previous studies reporting that CEE, in combination with GDNF, directs the fate of neural crest stem cells toward neurons[18] The observed upregulation of MAP2 in our study may be attributed to the AP-1 transcription factor, which harbors a binding site within the MAP2 promoter. Its activity can potentially be stimulated by certain components of CEE, such as thioredoxin. This hypothesis stems from our prior study, which identified several crucial components of CEE[19] In addition, our findings revealed a slight increase in β-III tubulin at passage 3. Although this increase was not statistically significant, its trend aligns with the expression pattern observed for MAP-2. Conversely, the expression of PDGFRα, a well-known marker of glial cells predominantly expressed at high levels in these stem cells, significantly decreased. This highlights a potential suppression of glial lineage commitment in these stem cells.

Furthermore, our results demonstrate elevated expression levels of BDNF and VEGF mRNA in response to CEE supplementation, whereas NGF transcript levels were decreased. Although the decreased NGF mRNA level may be accompanied by a stable or even increased protein level, as the transcription rate of NGF is much lower than the translation rate in mammals[48] this warrants further investigation in future studies. The enhanced neurotrophic profile of HFSCs after CEE supplementation may augment their therapeutic potential, as the paracrine effects of these stem cells have been shown to promote CNS recovery.[49,50] It is widely believed that the release of these factors may exert bilateral protective and/or regenerative effects on the injured CNS host tissue. It is worth mentioning that the observed upregulation of BDNF and VEGF may stem from the activation of CREB and c-Myc within these stem cells after CEE treatment.[19] Notably, CREB activation is triggered by active CamKII present in the CEE, whereas the regulation of the c-Myc transcription factor is governed by the signaling factor SMAD3, which is also found within CEE.[19]

Conclusion

In summary, our study reveals that preconditioning with CEE during the early stages of *in vitro* culture enhances the expression of the neuronal marker MAP2 and the trophic

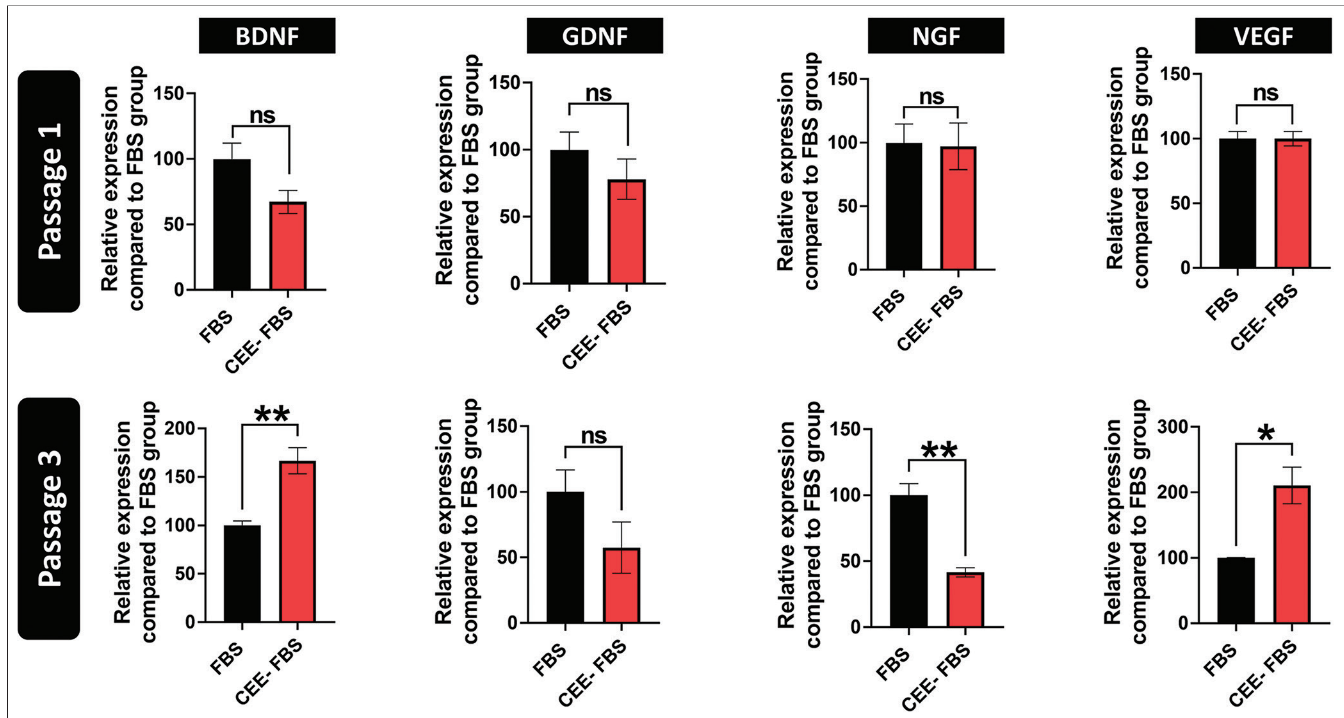

Figure 5: Expression profile of major trophic factors by hair follicle stem cells. Analysis of trophic and angiogenic factor transcripts indicates heightened levels of BDNF and VEGF in the CEE-FBS group, with unaltered GDNF expression and reduced NGF mRNA. The expression of target genes was normalized against the housekeeping gene HPRT. Values are mean $\pm$ SEM of three independent experiments (n = 3). A t-test was conducted to assess statistical differences. Significance levels are denoted as $*P < 0.05$ and $**P < 0.01$ compared with the FBS group

factors BDNF and VEGF, accompanied by morphological transformations from stellate to neuron-like shapes. These findings underscore the potential of CEE, with its diverse array of growth factors, to address certain limitations associated with HFSCs-based therapy, thereby enhancing its therapeutic efficacy and expanding its clinical applications. It's important to note that we cannot assert that neuronal differentiation is entirely complete. However, our gathered evidence strongly suggests that this preconditioning period can initiate and enhance their commitment toward the neuronal lineage.

Ethics approval and consent to participate

Animal care and protocols adhered strictly to the guidelines approved by the Animal Care Committee of Shiraz University of Medical Sciences, in accordance with internationally recognized ARRIVE guidelines. Furthermore, this study received approval from the Research Ethics Committees of Laboratory Animals at Shiraz University of Medical Sciences, with the approval ID: IR.SUMS.AEC.1401.086.

Availability of data and materials

The data that support the findings of this study are available on request from the corresponding author.

Financial support and sponsorship

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Conflicts of interest

There are no conflicts of interest.

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