



Research Article

The Effect of Retinoic Acid on the Expression of Nestin, GATA Binding Protein 3, Neurogenin 1, and Microtubule-Associated Protein 2 Markers in Mesenchymal Stem Cells Derived from Human Adipose Tissue

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Highlights

- One μM RA significantly elevated Nestin, NGN1, and MAP2 expression at day 14
- GATA3 upregulation was non-significant, suggesting late-stage transcriptional role
- RA monotherapy induced neurite-like morphology in hADSCs without extrinsic stimuli

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ABSTRACT

Background and Aim: Hearing loss in developing countries drives interest in stem cell therapies, though underlying molecular mechanisms remain partly unclear. One key pathway in this process is the Retinoic Acid (RA) signaling pathway. This study aimed to evaluate the effect of RA on the expression of neural markers, including nestin, Neurogenin 1 (NGN1), Guanine-Adenine-Thymine-Adenine Binding Protein 3 (GATA3), and Microtubule-Associated Protein 2 (MAP2), in human Adipose-Derived Stem Cells (hADSCs).

Methods: hADSCs were purchased and treated with RA (1 μM) under standard conditions for 7 and 14 days. RNA was extracted using the FavorPrep™ RNA kit and converted to complementary DNA (cDNA). Nestin, NGN1, GATA3, and MAP2 gene expression was assessed using real-time Polymerase Chain Reaction (PCR) with Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) as an internal control. Data analysis was conducted using the $2^{-\Delta\Delta Ct}$ method.

Results: RA significantly increased the expression levels of nestin, NGN1, and MAP2 after 14 days compared to the control group (day 0). RA also increased nestin and MAP2 expression after 7 days, although these changes were not statistically significant compared to the control; moreover, RA acid induced morphological changes in hADSCs.

Conclusion: This study demonstrated that RA enhances the neural differentiation of hADSCs by upregulating the neural markers nestin, NGN1, and MAP2. These findings highlight the potential role of RA in neural differentiation and its clinical applications. These findings suggest that RA's effects may be dose- and time-dependent, with one μM for 14 days enhancing neural marker expression.

Keywords: Auditory neurons; retinoic acid; stem cells; human adipose-derived stem cells

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Introduction

Hearing loss is the most common sensory impairment in humans, typically caused by damage to the hair cells of the cochlea or auditory neurons. While cochlear implants or hearing aids can be helpful in cases of minor damage, there is no effective treatment for auditory neuropathy [1]. Cell therapy is a promising strategy for replacing SGNs [1]. Understanding the molecular mechanisms that enhance the neural differentiation of stem cells into auditory neuron-like cells may contribute to the development of regenerative strategies in aural rehabilitation. Such approaches could potentially be used in conjunction with existing rehabilitation methods to enhance outcomes in patients with sensorineural hearing loss, particularly those with auditory neuropathy, for which conventional devices are less effective. Stem cells are considered a suitable source for cell therapy, as they are present in most differentiated tissues [2]. With their self-renewal capability, adult stem cells can maintain their division potential for extended periods and differentiate into various cell lineages [2]. Mesenchymal Stem Cells (MSCs), derived from bone marrow and other tissues, can be easily cultured *ex vivo* and differentiate into neural lineages [3]. Adipose-Derived Stem Cells (ADSCs) have been shown to express neurotrophic factors, including Nerve Growth Factor (NGF), Glial cell line-Derived Neurotrophic Factor (GDNF), and Brain-Derived Neurotrophic Factor (BDNF), *in vivo* [4]. Various studies demonstrate that ADSCs can produce specific neurotrophic factors and biologically active molecules that contribute to neuroprotection and nerve repair [5]. Nestin is recognized as a marker of neuroprogenitor cells in the central nervous system. It serves as an intermediate filament in neuroepithelial cells and neural precursors, which are expressed during early neurodevelopment [6]. Neurogenin 1 (NGN1) is crucial for the development of spiral ganglion neurons in the inner ear. In mice, the absence of NGN1 results in the complete loss of spiral ganglion neurons [7]. GATA3 is a critical transcription factor involved in neuronal development, particularly in the differentiation of auditory neurons and the formation of the auditory system [8]. Studies highlight the role of MAP2 in neural differentiation. A concentration of 1 μ M RA has been reported to increase the expression of neural-specific genes such as MAP2 and Pax family genes [9]. RA plays a pivotal role in the development

of the nervous system, exerting its effects on gene expression through nuclear receptors. Two types of RA receptors, Retinoic Acid Receptor (RAR) and Retinoid X Receptor (RXR), have been identified, with some of their expression induced by RA [10]. Studies on the viability of bone marrow stem cells after 72 hours of treatment revealed that RA concentrations of 0.5 and 5 μ M did not affect cell viability. In contrast, higher concentrations of 50 and 100 μ M significantly reduced it [11]. Research on mouse hair follicle stem cells exposed to RA indicated that concentrations above 10 μ M led to cell death through apoptosis or necrosis [12]. RA can inhibit cell proliferation by interfering with the progression of the cell cycle, particularly during the transition from the G1 phase (where cells prepare for DNA synthesis) to the S phase (where DNA replication occurs). It achieves this by downregulating key cell cycle-promoting regulators, such as cdk2, cyclin E, and cyclin A, and by upregulating anti-proliferative genes, including p27^{Kip1} and p16^{INK4A}, which act as cell cycle inhibitors that block the initiation of DNA synthesis [13]. Regarding the effects of RA on mesenchymal stem cells, studies have shown that RA concentrations of 0.25 and 0.5 μ M have minimal impact on cell growth or death. In contrast, doses of 1, 2, and 4 μ M significantly reduce cell viability [14]. Another study demonstrated that 10 μ M RA substantially decreases the viability of treated stem cells and induces apoptosis in these cells [15]. While high concentrations of RA induce cell death and apoptosis in stem cells, lower and appropriate concentrations can enhance the expression of genes and proteins that promote cell proliferation [16]. This study aimed to investigate the effects of RA at a concentration of 1 μ M on the expression levels of specific markers of auditory neuron differentiation (nestin, NGN1, GATA3, and MAP2) in human hADSCs, a dosage selected to promote neural differentiation while minimizing cytotoxic effects observed at higher concentrations.

Methods

Culture of human adipose-derived stem cells

hADSCs were obtained from the Royan Cell Therapy Institute (Tehran, Iran) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 15% Fetal Bovine Serum (FBS) and 1% streptomycin and penicillin. Once the flask was fully confluent, the cells were transferred to a 75 cm² flask. Subsequently, the cells were trypsinized, and 150,000 cells were seeded

into each well of a six-well plate that had been pre-coated with fibronectin. RA was added to the culture medium at a final concentration of 1 μ M. Day 0 refers to the time point at which RA treatment began. hADSCs were cultured in Ham's F-12 Nutrient Mixture (DMEM: F12) without FBS, and exposed to RA for 7 and 14 days to assess the time-dependent effects of RA on neural differentiation. Untreated hADSCs at day 0 (before RA exposure) served as the baseline control. Additionally, time-matched control groups were maintained under identical conditions without RA for 7 and 14 days, allowing direct comparisons with RA-treated cells. Since the differentiation medium lacked FBS, cell viability and proliferation were limited, resulting in a gradual decline in cell numbers over time. Therefore, extending cultures beyond 14 days was impractical, and extracting sufficient RNA for downstream analysis became increasingly complex. The 7- and 14-day time points were selected in line with most published neural differentiation protocols, which typically span one to three weeks. At the end of each time point, RNA extraction and gene expression analyses were performed. In addition to molecular analysis, morphological changes were observed and documented using phase-contrast microscopy ($\times 20$ magnification) on days 7 and 14. Morphological features suggestive of neural differentiation, such as extended processes resembling neurites, were recorded.

Gene expression analysis

Ribonucleic acid extraction

To minimize contamination, RNA extraction was conducted under sterile conditions using ultraviolet-irradiated hoods, 70% ethanol, and DEPC-treated solutions. Cryotubes, microtubes, pipette tips, and water used in the experiments were free from DNase and RNase. Additionally, all surfaces and gloves were decontaminated with DEPC solution to ensure RNase-free conditions. RNA was extracted from all cells, including the control (day 0) and treated groups, using the FavorPrep™ RNA Kit.

Ribonucleic acid quality analysis

The quality and concentration of RNA samples were assessed using a Nanodrop spectrophotometer by measuring Optical Density (OD) ratios at 260/280 nm and 260/230 nm. The A260/A280 ratios for the RNA samples ranged between 1.7 and 2.0, indicating high purity with minimal protein contamination. The

RNA concentration was quantified in micrograms per microliter (μ g/ μ L) for each sample.

Complementary deoxyribonucleic acid synthesis for gene expression analysis of nestin, neurogenin 1, guanine-adenine-thymine-adenine binding protein 3 and microtubule-associated protein 2

An equal amount of RNA (1 μ g) from each sample was used for complementary DNA (cDNA) synthesis to achieve uniform cDNA concentrations. cDNA is synthesized from RNA using reverse transcriptase. This process enables the analysis of gene expression, as cDNA serves as a stable and amplifiable template for quantitative PCR. cDNA was synthesized using a protocol optimized for a maximum RNA input of 5000 ng, although 1000 ng of RNA was used for each sample in this study. RNA samples were thawed on ice, while the cDNA synthesis kit reagents were equilibrated at room temperature. RNA was converted to cDNA under the following conditions: 25°C for 5 minutes, 42°C for 60 minutes, and 70°C for 5 minutes. The cDNA Revert Aid First Strand cDNA Synthesis Kit was employed for this purpose.

Real-time polymerase chain reaction for nestin, neurogenin 1, guanine-adenine-thymine-adenine binding protein 3 and microtubule-associated protein 2 genes

The real-time PCR reaction mixture consisted of the following components: 6 μ L SYBR Green Master Mix (2X) (YTA SYBR Green qPCR Master Mix 2X, Cat No: YT2551, final concentration 1X), 0.5 pmol forward primer (10 pmol/ μ L), 0.5 pmol reverse primer (10 pmol/ μ L), one μ L cDNA, and three μ L nuclease-free water. Each reaction was conducted in duplicate. The thermal cycling program for GATA3, nestin, NGN1, MAP2, and GAPDH was as follows: initial denaturation: 95°C for 5 minutes (1 cycle), denaturation: 95°C for 60 seconds (40 cycles), annealing: 60°C for 5 seconds, extension: 72°C for 5 seconds. A melt curve analysis was performed at the end of the real-time PCR run, with temperatures ranging from 72°C to 95°C, without a fixed duration (i.e., one cycle). Table 1 presents the specific primer sequences used for amplifying neural marker genes in this study.

Data analysis

The experiments were performed with three biological replicates and three technical replicates. Data normality was assessed using the Shapiro-Wilk test, which indicated that the data did not meet the assumptions of normality.

Table 1. Primer sequences for neural marker gene analysis

Gene	Sequence (5'→3')	Length	Product length	Identification code
MAP2	F: GGAGTAACCAAGAGCCCAGAAAAG	25	262 bp	>XM_017004140.1
	R: GAGTGCCTGGTGTGCGTGAAGA	22		
GATA3	F: GACGGTCAAGGCAACCACG	19	131 bp	>NM_003106.3
	R: CCAGGGTAGGGATCCATGAAGC	22		
Nestin	F: CACCCCTCAGCCCTGACCACT	21	135 bp	>NM_006617.1
	R: CCTCTATGGCTGTTTCTTCTCTACCA	28		
Ngn1	F: AAACGAGCATGAAAATTCGGT	21	82 bp	>NM_006161.2
	R: ATGAAGCCCACTCCTTGCT	20		
GAPDH	F: GAGTCCACTGGCGTCTTCAC	20	110 bp	>NM_001289745.2
	R: ATGACGAACATGGGGGCATC	20		

MAP2; microtubule-associated protein 2, GATA3; guanine-adenine-thymine-adenine binding protein 3, Ngn1; neurogenin 1, GAPDH; glyceraldehyde-3-phosphate dehydrogenase

Therefore, the non-parametric Kruskal-Wallis H test was used to compare gene expression levels between groups. Following the statistical analysis, Dunn's post hoc test was applied to identify significant pairwise differences between time points. Data analysis was conducted using SPSS software version 17 and GraphPad Prism. Final graphs were generated using GraphPad Prism version 8.8. A P-value less than 0.05 was considered statistically significant.

Results

Evaluation of gene expression for nestin, neurogenin 1, guanine-adenine-thymine-adenine binding protein 3 and microtubule-associated protein 2

The Kruskal-Wallis test demonstrated statistically significant differences in expression levels of nestin ($H=7.44$, $p=0.003$), NGN1 ($H=7.26$, $p=0.003$), and MAP2 ($H=6.93$, $p=0.007$) across the three time points, indicating that these genes are significantly regulated over time following RA treatment. However, the change in GATA3 expression did not reach statistical significance ($H=5.53$, $p=0.05$).

Nestin gene expression at different treatment times

This study demonstrated that the mRNA expression of the nestin gene in the RA-treated groups increased after 7 and 14 days compared to day 0 (untreated control), with this difference being statistically significant on day

14 ($p=0.003$). RA treatment resulted in a 3.03-fold and 7.98-fold increase in Nestin gene expression on days 7 and 14, respectively, compared to the control group (Figure 1).

RA treatment significantly increased nestin gene expression on days 7 and 14 compared to day 0 (untreated control), as determined by the Kruskal-Wallis test ($p=0.003$). To further explore group differences, Dunn's post hoc test was conducted, revealing a

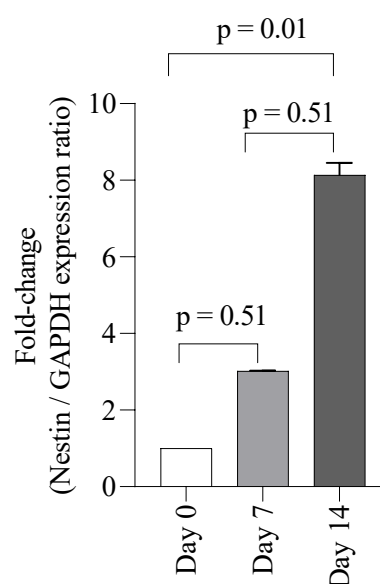


Figure 1. Nestin gene expression on different days post-treatment with 1 μ M retinoic acid. GAPDH; glyceraldehyde-3-phosphate dehydrogenase

significant increase in expression between day 0 and day 14 ($p=0.01$). Gene expression values are reported as mean±Standard Error of the Mean (SEM).

Neurogenin 1 gene expression at different treatment times

The results indicated that mRNA expression of the NGN1 gene in the RA-treated groups increased after 7 and 14 days compared to day 0 (untreated control), with this difference being statistically significant only after 14 days ($p=0.003$). RA treatment resulted in a 1.06-fold and 6.76-fold increase in NGN1 gene expression on days 7 and 14, respectively, compared to day 0 (untreated control) (Figure 2).

Expression levels increased on days 7 and 14 compared to Day 0 (untreated control), with a statistically significant increase observed on day 14 as determined by the Kruskal-Wallis test ($p=0.003$). Subsequently, Dunn's post hoc test was applied to identify significant pairwise differences between time points, revealing a substantial difference between day 0 and day 14 ($p=0.02$). Data are presented as mean±SEM.

Guanine-adenine-thymine-adenine binding protein 3 gene expression at different treatment times

The results revealed that RA increased mRNA expression of the GATA3 gene in the treated group after 14 days; however, this difference was not statistically

significant ($p=0.05$). Additionally, RA did not affect the expression of GATA3 after 7 days (Figure 3).

Although expression levels increased on day 14 compared to day 0 (untreated control), the overall difference among groups was not statistically significant (Kruskal-Wallis test, $p=0.05$), and Dunn's post hoc test did not identify substantial pairwise differences. Data are presented as mean±SEM.

Microtubule-associated protein 2 gene expression at different treatment times

The results indicated that mRNA expression of the MAP2 gene in the RA-treated groups increased after 7 and 14 days compared to Day 0 (untreated control). Still, this difference was statistically significant only on day 14 ($p=0.007$). RA treatment resulted in a 1.36-fold and 2.31-fold increase in MAP2 gene expression on days 7 and 14, respectively, compared to the control group (Figure 4).

Expression levels increased on days 7 and 14 compared to day 0 (untreated control), with a statistically significant increase observed on day 14 as determined by the Kruskal-Wallis test ($p=0.007$). Subsequently, Dunn's post hoc test was applied to identify significant pairwise differences between time points, revealing a substantial difference between day 0 and day 14 ($p=0.02$). Data are presented as mean±SEM.

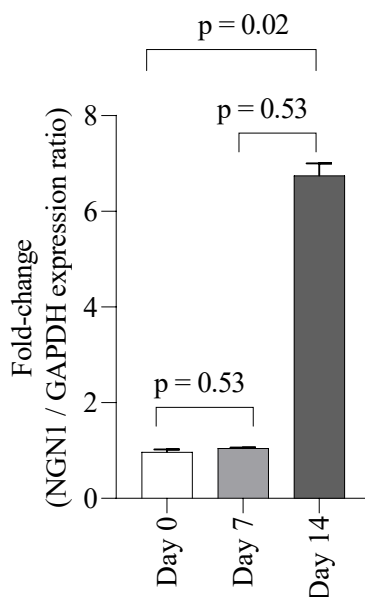


Figure 2. Neurogenin 1 gene expression after treatment with 1 μ M retinoic acid. NGN1; neurogenin 1, GAPDH; glyceraldehyde-3-phosphate dehydrogenase

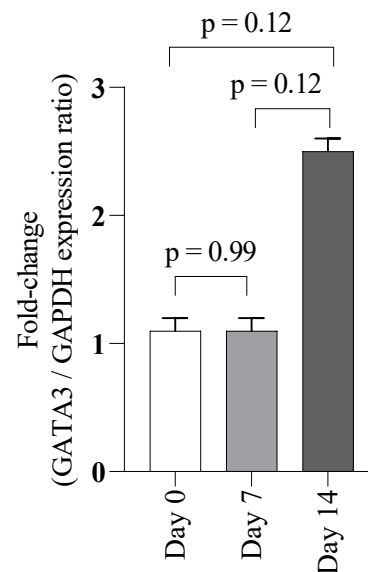


Figure 3. Guanine-adenine-thymine-adenine binding protein 3 gene expression at different time points following 1 μ M retinoic acid treatment. GATA3; guanine-adenine-thymine-adenine binding protein 3, GAPDH; glyceraldehyde-3-phosphate dehydrogenase

Morphological changes following retinoic acid treatment

Microscopic evaluation of hADSCs after RA treatment (1 μ M) revealed distinct morphological changes indicative of neural differentiation. Treated cells exhibited elongated processes resembling neurites, consistent with a neurogenic morphology. These changes were observed on days 7 and 14 under phase-contrast microscopy.

Figure 5 illustrates the morphological changes observed in hADSCs post-exposure to RA under a magnification of X20. The treated cells exhibit

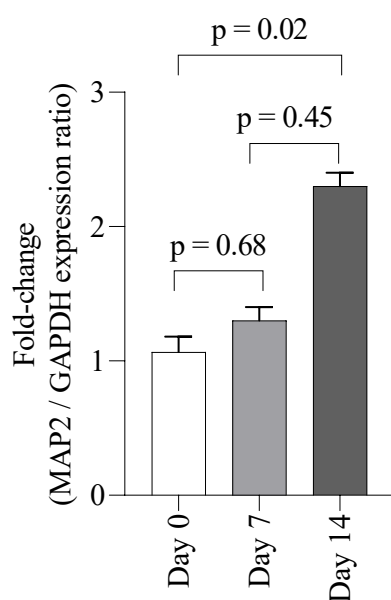


Figure 4. Microtubule-Associated Protein 2 gene expression after treatment with 1 μ M retinoic acid. MAP2; microtubule-associated protein 2, GAPDH; glyceraldehyde-3-phosphate dehydrogenase

alterations characteristic of neural differentiation, including extended cellular processes resembling neurite-like structures.

Discussion

This study provided compelling evidence that RA at a concentration of 1 μ M promotes the neural differentiation of hADSCs by significantly upregulating the expression of neural markers, including nestin, NGN1, and MAP2. These findings confirm previous studies while adding insights into the temporal gene expression profile induced by RA. Although GATA3 expression also increased on day 14, the change did not reach statistical significance, possibly reflecting its role in later stages of auditory neurogenesis [8]. RA treatment led to a significant upregulation of nestin on days 7 and 14. Nestin is a well-established marker of neural progenitor cells and early commitment to the neuroepithelial lineage [6]. These results are consistent with studies by Homayouni Moghadam et al. [17], Esfandiari et al. [18], and Xu et al. [19], which demonstrated increased Nestin expression following RA exposure in mouse embryonic stem cells and hADSCs. Collectively, these data suggest that RA facilitates early neural lineage commitment. NGN1, a key transcription factor for auditory neuron development, was significantly upregulated after 14 days of RA treatment. NGN1 plays a crucial role in the development of spiral ganglion neurons [7], and its late induction suggests that prolonged RA exposure supports the early neurogenic specification pathway necessary for the regeneration of auditory neurons. MAP2, a marker of mature neurons, also showed a significant increase on day 14. This finding is in

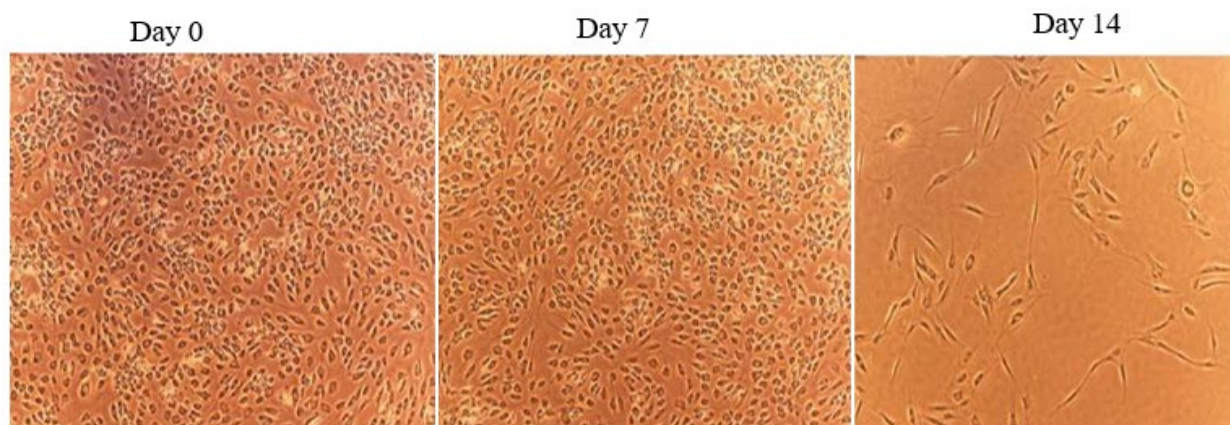


Figure 5. Morphological changes in human adipose-derived stem cells following treatment with 1 μ M retinoic acid were observed under phase-contrast microscopy ($\times 20$).

agreement with the results of Asgari et al. [20], Xu et al. [19], and Esfandiari et al. [18], who reported that RA alone or in combination with other factors upregulates MAP2 expression. These observations suggest that RA promotes not only the induction of neural progenitors but also the progression toward more mature neuronal phenotypes. In contrast, the upregulation of GATA3 was modest and not statistically significant. Given that GATA3 plays a critical role in the organization of the auditory system during later developmental stages [8], the 14-day treatment period may not have been sufficient to induce significant expression. Further studies with more extended differentiation periods are warranted to investigate GATA3's regulatory dynamics more thoroughly. Despite using only RA, without nanomaterials, scaffolds, or growth factor cocktails, our study achieved comparable neural marker expression to more complex protocols. For example, Esfandiari et al. [18] utilized aligned nanofibrous scaffolds and growth factors, while Asgari et al. [20] employed RA conjugated with gold nanoparticles. Our findings suggest that one μM RA alone is sufficient to induce substantial neural differentiation in hADSCs under defined conditions. These effects appear to be both dose- and time-dependent. Toxicological studies have shown that RA concentrations above 10 μM can reduce cell viability and induce apoptosis [11, 12, 21], whereas concentrations such as one μM maintain viability while promoting differentiation [14, 16]. This concentration, therefore, represents a balance between neuroeducation and cytocompatibility. Clinically, these results suggest the potential of RA-induced hADSCs in developing cell-based therapies for sensorineural hearing loss and auditory neuropathy, where traditional approaches, such as cochlear implants, are insufficient [1]. Moreover, combining RA with neurotrophic factors, such as BDNF or GDNF [22], or integrating it with nanofiber scaffolds [20, 22] may further enhance the neural differentiation potential. Future research should aim to extend the duration of RA exposure, evaluate protein-level expression using immunocytochemistry, assess electrophysiological functionality, and explore the use of RA in combination with bioengineered delivery platforms. These steps are necessary to translate the *in vitro* findings into applicable regenerative strategies. In conclusion, this study substantiates the role of RA as a potent inducer of neural marker expression in hADSCs, providing a foundation for optimizing

differentiation protocols for therapeutic applications in neural tissue engineering and the repair of the auditory system. Moreover, from a rehabilitation standpoint, the use of RA-treated hADSCs may offer future avenues for restoring neuronal networks or replacing damaged auditory neurons, particularly when combined with existing therapies such as cochlear implants or auditory training. At the same time, this cellular model provides a valuable platform for basic science research to dissect the molecular mechanisms by which RA and neurotrophic factors regulate gene expression and lineage commitment in hADSCs. Further research is recommended to explore the combined effects of RA with other factors.

Conclusion

This study demonstrated that Retinoic Acid (RA) at a concentration of 1 μM effectively induces neural differentiation in human Adipose-Derived Stem Cells (hADSCs), as evidenced by the significant upregulation of key neural markers, including nestin, neurogenin 1, and microtubule-associated protein 2, particularly after 14 days of treatment. While guanine-adenine-thymine-adenine binding protein 3 expression showed a slight increase, it did not reach statistical significance, suggesting the need for extended exposure or additional differentiation signals to activate late-stage neural transcription factors. The findings confirm that RA alone, without the aid of external scaffolds or growth factors, is sufficient to promote neural lineage commitment under defined conditions. Notably, the observed changes in gene expression were time-dependent, underscoring the critical role of duration in differentiation protocols. These results highlight the potential of RA-treated hADSCs as a promising cellular model for neural tissue engineering and regenerative medicine, particularly in addressing auditory neuron damage associated with sensorineural hearing loss. Furthermore, this model can serve as a valuable *in vitro* system for investigating the molecular mechanisms underlying RA-induced neural differentiation. Future studies are encouraged to explore the synergistic effects of RA with neurotrophic factors and nanomaterials, validate neural phenotypes at the protein and functional levels, and evaluate therapeutic efficacy in preclinical models. Overall, RA represents a potent and practical agent for neural induction, offering promising implications for both clinical application and basic neuroscience research.

Ethical Considerations

Compliance with ethical guidelines

The Ethics Committee of Shahrekord University of Medical Sciences approved this study under the ethical code IR.SKUMS.REC.1397.307. This study was conducted under the Declaration of Helsinki.

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Data availability statement

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Authors' contributions

MRMS: Drafting the manuscript, study design, finalized the submission and statistical analysis; SL: Conceptualization and data collection; KA: Interpretation of the results and conceptualization; AS: Contributed to the study design and data collection; RY: Analysis and interpretation of data and editing; GRM: Supervised the study and wrote the main manuscript text.

Conflict of interest

The authors declare no conflict of interest.

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