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Adipose-Derived Mesenchymal Stem Cell (AD-MSC)-Like Cells Restore Nestin Expression and Reduce Amyloid Plaques in Aluminum Chloride ($AlCl_3$)-Driven Alzheimer's Rat Models

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Background: Alzheimer's disease (AD) is a neurodegenerative disorder with a significant burden on public health, and current treatments offer limited efficacy. This study investigated the effectiveness of adipose-derived mesenchymal stem cells (AD-MSCs) on the expression of the nestin gene and amyloid plaque in an aluminum chloride ($AlCl_3$)-driven Alzheimer's rat model.

Materials and methods: AD-MSCs were characterized using flow cytometry. Adult male Wistar rats were treated with/without $AlCl_3$ and injected with/without AD-MSCs. After 5 days of $AlCl_3$ ingestion and 4 weeks of subsequent AD-MSCs intraperitoneal injection, behavioral and molecular assessments were conducted. The Y-maze alternation test was used to test spatial learning of rats. Nestin gene expression was evaluated using reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The presence of amyloid plaque in the cortex and the hippocampus was evaluated through Congo red staining.

Results: AD-MSC-like cells expressed the MSC markers CD90, CD73 and CD105. The Y-maze alternation result for rats treated with $AlCl_3$ and AD-MSC-like cells was significantly higher compared with rats treated with $AlCl_3$ only. Nestin gene expression was significantly higher in rats treated with $AlCl_3$ and AD-MSC-like cells compared to those treated with $AlCl_3$ only. After AD-MSC-like cells treatment, the Congo red staining results of rat's cortex and hippocampus were significantly decreased.

Conclusion: The findings suggest that AD-MSC-like cells possess therapeutic potential in restoring neural plasticity, amyloid plaque clearance and warrant further investigation for AD treatment. This study contributes to the emerging field of stem cell therapy for neurodegenerative diseases by highlighting the promise of AD-MSCs.

Keywords: *Alzheimer's disease, adipose-derived mesenchymal stem cells, neural plasticity, Congo red staining, stem cell therapy*

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Introduction

Alzheimer's disease (AD), a neurodegenerative condition, severely affects memory, cognition, and day-to-day functioning. The disease, which affects millions of individuals globally, is characterized by the buildup of beta-amyloid plaques and neurofibrillary tangles. It has a significant social and economic impact.[1-3] In parallel, aluminum chloride ($AlCl_3$) ingestion has been studied as an environmental factor that may exacerbate Alzheimer's symptoms, as it tends to accumulate in brain tissue, thereby possibly affecting cognitive function.[4-6]

Recent studies have shifted focus toward the potential of stem cell therapy for neurodegenerative diseases, with adipose-derived mesenchymal stem cells (AD-MSCs) gaining particular attention.[7] These cells have been demonstrated to have neuroprotective and immunomodulatory effects, but their role in modulating specific genes involved in Alzheimer's remains underexplored.

The novelty of the current study lies in exploring the influence of AD-MSCs on the expression of nestin, a protein crucial for neurogenesis, in AD models induced by aluminum chloride ingestion. Administration of mesenchymal stem cells (MSCs) to amyloid treated neural progenitor cells significantly increased the expression of glial fibrillary acidic protein (GFAP), Ki67, HuD, nestin and sex determining region Y-box 2 (SOX2). The number of nestin-expressing cells showed a significant increase in the hippocampal dentate gyrus in the AD group compared with the control group.[8] After transplantation of bone marrow-derived MSCs (BM-MSCs) into Alzheimer's mice, there was an increase in Nestin gene expression.[9] However, there was a statistically significant decrease in nestin in the dentate gyrus in the group with Alzheimer's compared to controls.[10]

Most existing studies either focus on the effects of stem cells in Alzheimer's treatment without specific gene modulation or study gene modulation without the context of stem cell treatment.[11,12] This study aims to bridge this gap by providing insights into the genetic modulation capabilities of AD-MSCs in an $AlCl_3$-induced Alzheimer's model, thereby extending the scope of stem cell therapy for AD. The primary objective of this study was to investigate the effect of AD-MSCs on the amyloid plaque accumulation and the nestin genesexpression in $AlCl_3$-induced AD in rats.

Materials and methods

Experimental Animals and Housing Conditions

A total of 15 male adult Wistar rats were procured from the Animal House of the Biomedical Laboratory, Faculty of Medicine, Universitas Andalas, Indonesia. The rats had an average ranging between 200 and 250 grams. The animals were housed in carefully regulated conditions, including a 12-hours light/dark cycle, a temperature of 23°C, and a humidity level of 60%. Before implementing any experimental interventions, the animals underwent a one-week acclimation period. During this time, the animals had unrestricted access to water and were fed a conventional rat diet. The study's technique and procedures were granted clearance by the Research Ethics Committee of the Faculty of Medicine, Universitas Andalas, Indonesia (No. 1093/UN.16.2/KEP-FK/2022). Considerable effort was made to minimize the distress endured by the animals.

AD-MSCs Culture and Characterization

AD-MSCs were obtained from the Indonesian Medical Education and Research Institute (IMERI), Faculty of Medicine, Universitas Indonesia. A complete medium consisting of alpha modified eagle medium (α-MEM) high glucose, 1% penicillin/streptomycin, and 10% fetal bovine serum (FBS) was utilized to culture the cells. Then, the cells were incubated at 37°C with 5% CO_2 for 3 days. Subculturing was performed using 0.25% trypsin-ethylenediamine tetraacetic acid (EDTA) solution. The culture medium was changed every 3-4 days until the cells were ready for use, at passage 3.[13-15] Flow cytometry was employed to validate the identification of AD-MSCs by assessing the presence of surface markers specific to MSCs, namely CD73, CD90, and CD105.[16] The tube containing cell suspension was centrifuged at 500 xg for 5 minutes. Then, the supernatant was removed, and the pellet was collected and labeled with CD73, CD90, and CD105 antibodies by adding 2.5 µL of each antibody to the tube containing the pellet. Afterward, the antibodies were homogenized with the pellet using a 10 µL pipette using the up-and-down technique and then incubated for 20 minutes in a dark room. The mixture was then vortexed briefly and analyzed using BD FACSAria III flow cytometer at 50,000-100,000 cells/second event. FACS Diva 8.0 software was used to determine the gating of AD-MSCs population that were not incubated with antibodies.

$AlCl_3$ Induction

The rats were divided into 3 groups (n=5 per group) using a simple randomization method. AD was induced in the experimental groups with administration of $AlCl_3$ (Merck, Darmstadt, Germany), while the sham group remained untreated. The rats were subjected to oral administration of $AlCl_3$ for a continuous period of 5 days, at a dosage of 300 mg/kg of body weight (BW). $AlCl_3$ was dissolved in 1 mL of distilled water per 100 kg of rat weight.[16] The experimental design included 3 groups to assess the interventions' effects: the phosphate-buffered saline (PBS) group, which received PBS but no specific intervention; the $AlCl_3$+PBS group, which was exposed to aluminum chloride ($AlCl_3$) and treated with PBS, serving as a baseline for $AlCl_3$ effects; and the $AlCl_3$+AD-MSCs group, which was subjected to $AlCl_3$ and treated with AD-MSCs.

Intraperitoneal Injection of AD-MSC-like Cells

To induce anesthesia, all groups were subjected to intraperitoneal injections of 15 mg/kg xylazine (Merck) and 50 mg/kg ketamine (Merck).[18] The rats in the $AlCl_3$+AD-MSCs group were subjected to intraperitoneal injections of AD-MSCs at a dosage of $1x10^6$ cells per rat.[17] The other groups were administered an equal volume of PBS (Merck) per rat. Following one-month duration subsequent to the AD-MSCs injection, the animals were sacrificed using cervical dislocation. The brain tissue was aseptically exposed, dissected, and removed. The brain tissue of the rat was then placed into film bottles.

Y-maze Alternation Test

Following one-month duration subsequent to the administration of AD-MSCs and before sacrifice, the rats were subjected to a Y-maze alternation test. The maze was divided into three equally spaced parts. Each rat was given full access to explore for a period of 8 minutes. The series of arm entries was recorded, and the percentages of alternation were calculated to evaluate spatial memory. After each trial, the maze was cleaned with 70% ethanol to eliminate residual odors and mitigate the influence of olfactory cues on subsequent rodents. The ethanol was allowed to fully evaporate before introducing the next rat.

To minimize bias, Y-maze arm entries were recorded via video capture instead of direct observation. Cameras were deliberately placed at multiple angles to ensure extensive coverage of all three arms, allowing thorough analysis of the recorded footage from a remote location. The camera footage was subsequently analyzed using specialized tracking software, DeepLabCut (available at https://github.com/AlexEMG/DeepLabCut), to ensure the precision of the sequences.[10,19]

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

Total RNA from the brain tissues of all groups was isolated using the TRIzol® reagent (Merck) and chloroform, followed by precipitation with 2x isopropanol (Merck). The RNA pellets were then washed with 70% ethanol. The pellets were resuspended in RNAse-Free Water (Merck). After that, RNA was quantified and equalized at 1,000 ng.

The composition of total cDNA synthesis was 5 µg total RNA, 1x RT buffer, 20 pmol oligodT, 4 mM dNTP, 10 mM dithiothreitol (DTT), 40 U SuperScriptTM II RTase enzyme (Invitrogen, Carlsbad, CA, USA), and Nuclease Free Water with a reaction volume of 20 µL. Total cDNA synthesis was carried out at 52°C for 50 minutes according to the manufacturer's instructions.

After completion of cDNA synthesis, qPCR was performed using primers that were optimized for design and temperature (Table 1).[20] SensiFAST™ SYBR® No-ROX Kit (Bioline, London, UK) was used to amplify the target genes according to manufacturer's instructions. qPCR was conducted for 40 amplification cycles consisting of a pre-denaturation step at 95°C for 3 minutes, an initial denaturation at 94°C for 5 minutes, a core cycle of denaturation at 94°C for 45 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 45 seconds, then final extension at 72°C for 7 minutes.

Table 2. Primer Sequences for RT-qPCR

Gene	Type	Primer Sequence (5'-3')
GAPDH	Housekeeping gene	F-TCAACAGCAACTCCCACTCTTCCA
		R-ACCCTGTTGCTGTAGCCGTATTCA
Nestin	Neural stem cells	F- GAGGTGGCTACATACAGGACTC
		R- AAGAGAAGCCTGGGAACCTC

Congo Red Staining

Rat brain tissues were soaked in 8% HNO_3. The tissues were dehydrated, embedded in liquid paraffin, and allowed to solidify. The paraffin blocks were then sectioned using a microtome with a thickness of 5 µm. The ribbon-shaped

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sections were placed on warm water at a temperature of 45°C and lifted onto a glass object coated with albumin solution as an adhesive for the preparation. The sections were dried and stained with Congo red. The proportion of amyloid deposits was calculated using the ImageJ program (ImageJ 1.49v software, National Institute of Health, Bethesda, MD, USA) by isolating the stained regions. Deposits were reported as a proportion of the total area.

Statistical Analysis

The results were presented as the mean and standard error of the mean (SEM). One-way analysis of variance (ANOVA) and Tukey post hoc test were used in the statistical analysis using SPSS software (IBM, Armonk, NY, USA) version 26.

Results

Characterization of AD-MSC-like cells

Based on the flow cytometric results, the cells were found to highly express the CD73, CD90, and CD105 markers (Figure 1). The percentages of CD73, CD90, and CD105 expression were 99.6%, 98.5%, and 99.4%, respectively. Conversely, the absence of CD14, CD19, CD45, and human leukocyte antigen - DR isotype (HLA-DR) markers indicated that these cells did not express these markers.

Spatial Learning Test

The Y-maze test was utilized in the behavioral investigation to evaluate the proclivity of rats to engage in an exploration of unknown situations, as evidenced by the outcomes of the research. Typically, rats demonstrate a proclivity for exploring a novel arm of the maze rather than revisiting a previously acquainted one. A discernible reduction in the frequency of entries into the desired arm was observed when comparing $AlCl_3$+PBS group with both the PBS and $AlCl_3$+AD-MSCs group (Figure 2, Figure 3).

Congo Red Staining

The Congo red staining results of rat's cortex were decreased significantly (p=0.023). There was a significant difference in the stained areas proportion between the PBS and the $AlCl_3$ +PBS groups (p=0.035), as well as between the $AlCl_3$+PBS and $AlCl_3$+AD-MSCs groups (p=0.043). Meanwhile, there was no significant difference in Congo red staining results between the PBS group and the $AlCl_3$+AD-MSCs group (p=0.556). However, the Congo red staining results of rat's hippocampus treated with AD-MSC-like cells were decreased, but not significant (p=0.060). The results were more pronounced in the cortex than in the hippocampus, indicating regional variations in the effectiveness of AD-MSCs based treatment that warrant further investigation.

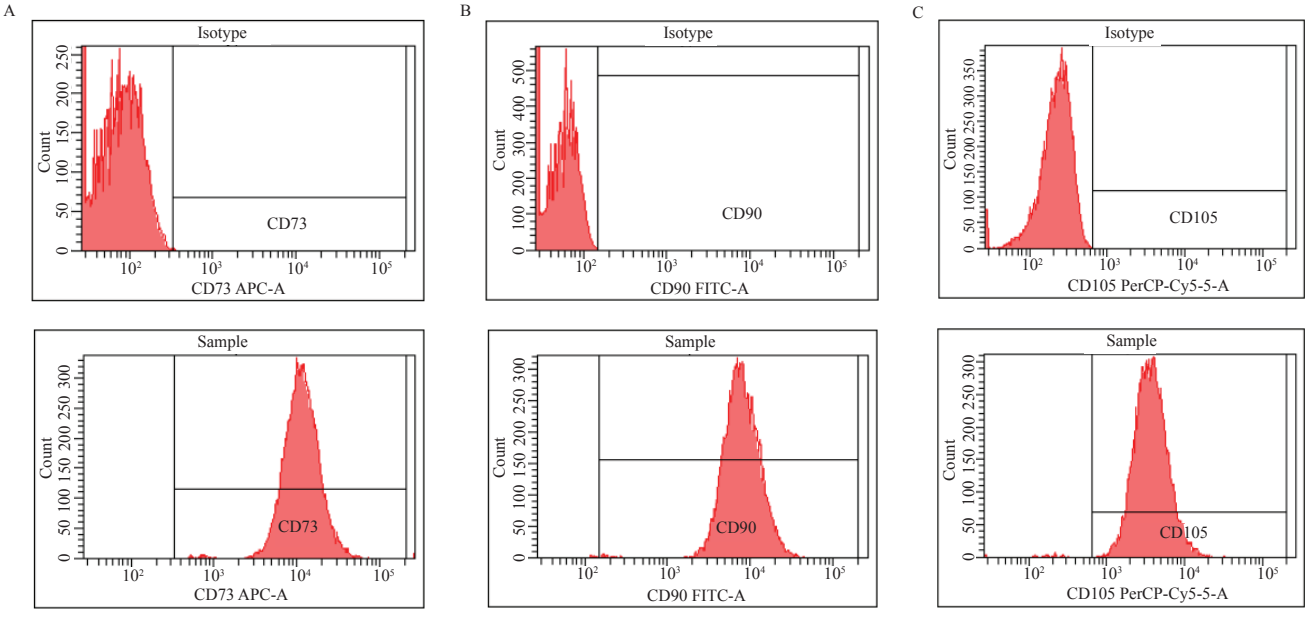

Figure 1. Flow cytometric results of AD-MSC-like cells. The flow cytometric analysis of AD-MSC-like cells demonstrated the presence of CD73, CD90, and CD105 markers.

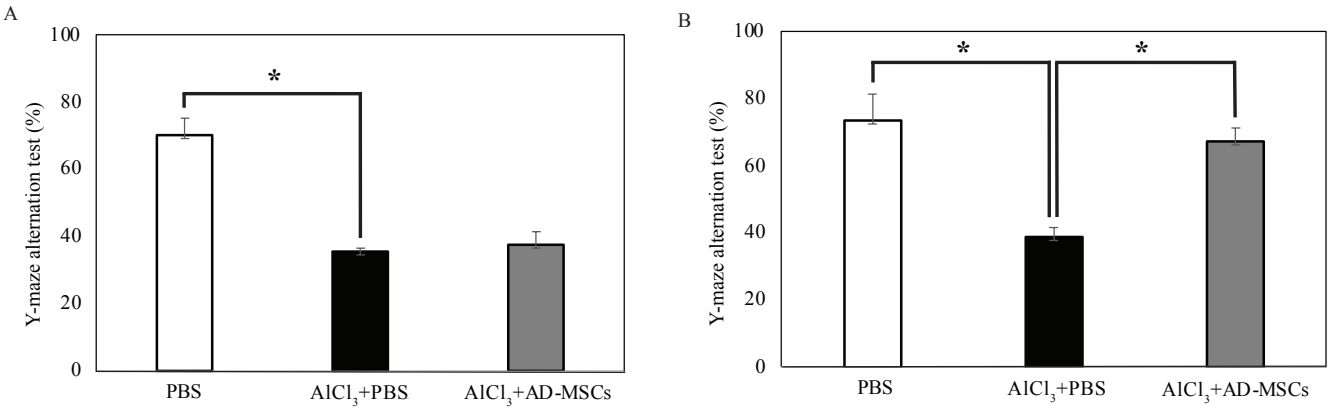

Figure 2. Percentages of Y-maze alternation test, (a) before and (b) after AD-MSC-like cells treatment. The data was presented as mean±SEM. The percentages were subjected to Tukey's post hoc analysis (*p<0.001).

Nestin Gene Expression

In rats from the $AlCl_3$+PBS group, neural progenitor marker nestin was significantly less expressed compared to the PBS group (Figure 4). Treatment with AD-MSC-like cells in the $AlCl_3$+AD-MSCs group increased nestin expression. The difference in nestin expression between the $AlCl_3$+PBS group and both the PBS group (p=0.001) and the $AlCl_3$+AD-MSCs group (p=0.001) was statistically significant. However, there was no significant difference between the PBS group and the $AlCl_3$+AD-MSCs group (p=0.983).

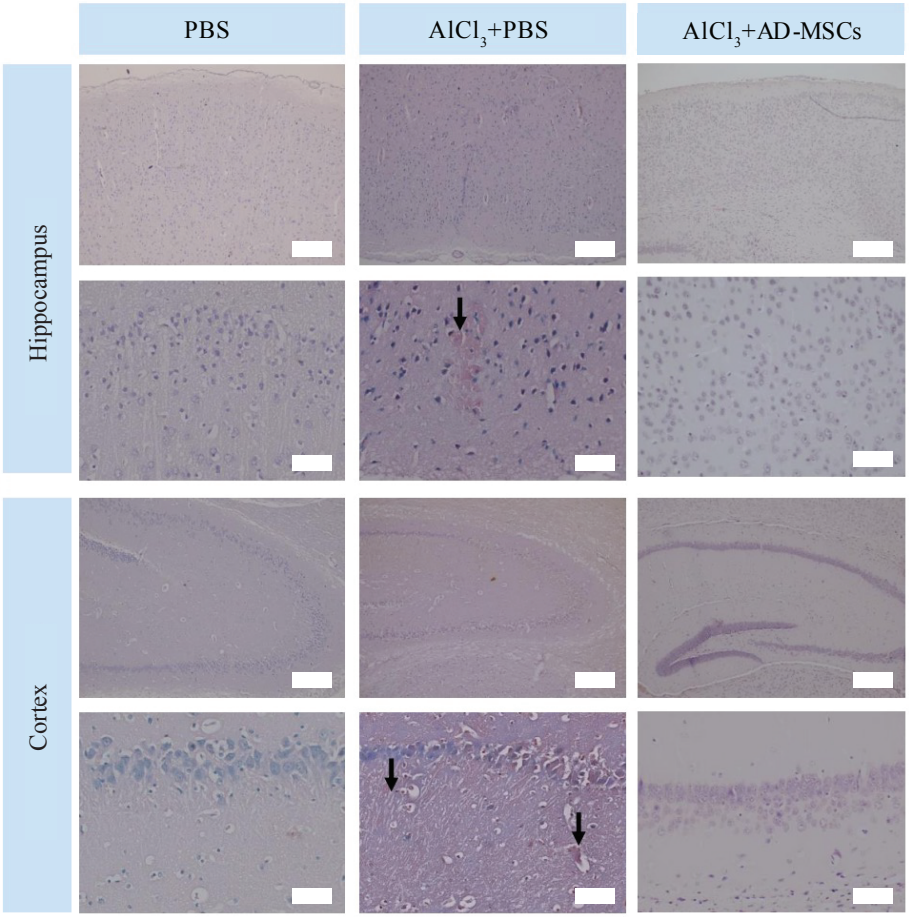

Figure 3. Calculation of amyloid deposit proportions in brain tissue of the rats using Congo red staining. Areas in the cortex and hippocampus of brain from the PBS, $AlCl_3$+PBS, $AlCl_3$+AD-MSC groups were shown. Amyloid plaque was detected as a reddish-orange material in the extracellular matrix and, in some cells, within the cytoplasm. A reduction in amyloid deposits was observed in treatments involving MSCs. White bar: 50 µm.

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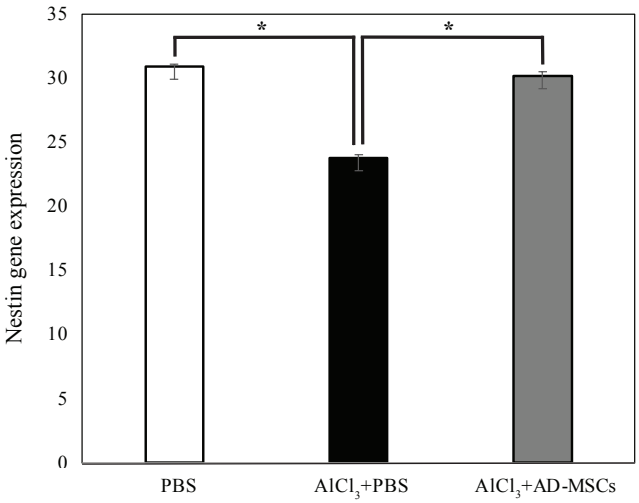

Figure 4. Nestin gene expression of the brain of $AlCl_3$-induced AD rat model. One-way analysis of variance (ANOVA) followed by the Tukey's post hoc test was used to evaluate significant differences between groups.

Discussion

The study's findings suggest a compelling direction in the search for viable AD treatments, particularly in scenarios where $AlCl_3$ ingestion is a contributory factor. Among MSCs, AD-MSCs appear to be particularly suitable for clinical applications. Adipose tissue is known to contain cells with high proliferative capacity *in vitro* and the ability to differentiate into multiple cell lineages. Because adipose tissue is easily accessible, autologous transplantation of AD-MSCs transplantation, like other MSCs, can be safely performed without risk of immune rejection or tumorigenesis. It has also been reported that AD-MSCs secrete growth factors including vascular endothelial growth factor (VEGF), glial cell-derived neurotrophic factor (GDNF), neurotrophin 3 (NT-3), nerve growth factor (NGF) and basic fibroblast growth factor (bFGF), and to participate in immunization by regulating cytokine release.[21-23] In this context, the nearly identical nestin gene expression levels between the PBS group and the $AlCl_3$+AD-MSCs group are remarkable. This near-normalization suggests that AD-MSC-like cells might have the capability to counteract or repair the neurodegenerative effects instigated by $AlCl_3$. The statistical significance further emphasizes that these results are unlikely to be due to random variations and are worthy of further exploration.

Nestin, a type VI intermediate filament protein, is considered a neural stem cell marker and is usually upregulated during neural tissue repair. It plays a critical role in cell survival and the differentiation of neural cells. The significant increase in nestin gene expression in the $AlCl_3$+AD-MSCs group hints at the potential of AD-MSC-like cells to trigger neural repair mechanisms.[11,18,24] Further bolstering the value of AD-MSCs in neurogenesis is their interaction with the local microenvironment, where they secrete neurotrophic factors and cytokines that could contribute to neural repair.[25,26] The low nestin expression in the group which received only $AlCl_3$ adds another layer to the study. $AlCl_3$ is known for its potential role in exacerbating AD symptoms.[4] The reduced nestin gene expression in this group indicates a detrimental effect on neural cells, supporting previous literature that highlights the neurotoxic effects of aluminum compounds.[6]

The use of Congo red staining for visualization of amyloid plaques has long been standard in AD research due to its sensitivity and specificity. The hippocampus is often considered the most severely affected site during AD development.[27] It is rich in glutamatergic, cholinergic, and monoaminergic axon terminals, and abnormalities in these

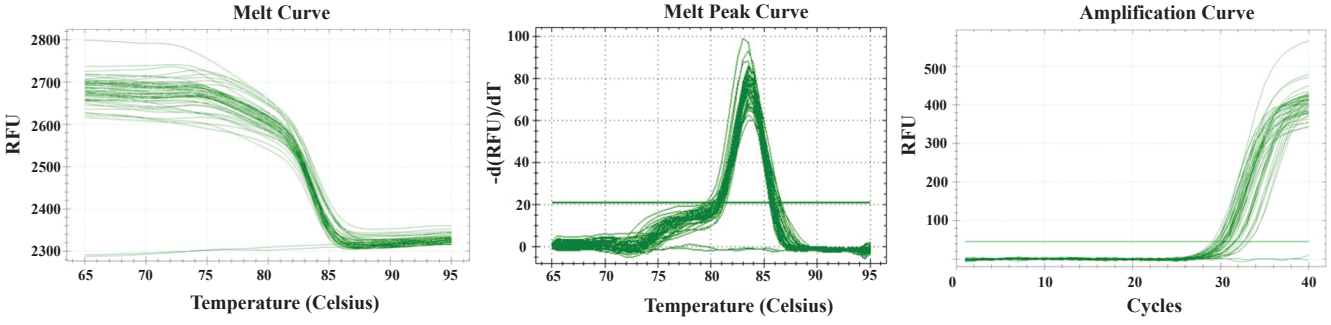

Figure 5. RT-qPCR results of nestin gene expression.

neurotransmitters are frequently reported to be associated with AD.[28] The use of AD-MSC-like cells has also been explored. AD-MSC-like cells not only reduced amyloid plaques but also improved cognitive function in AD model mice. Although the primary focus of this study was on plaque accumulation, the findings highlight the potential holistic effects of AD-MSCs in AD treatment.[29] The US Food and Drug Administration has approved only five types of drugs for the clinical treatment of AD: the cholinesterase inhibitor tacrine, galantamine, donepezil, rivastigmine, and the glutamate receptor antagonist memantine.[30,31] However, these five pharmacological agents can only relieve AD symptoms; they do not stop or reduce AD brain pathology, nor do they stop disease progression. The findings of the current study have significant implications for the development of stem cell therapies for AD. Given that $AlCl_3$ ingestion is considered an environmental risk factor for AD, the protective role of AD-MSCs in this context adds another layer to our understanding of how stem cell therapies could be employed in treating neurodegenerative disorders. However, the study also raises questions for future research. For instance, it is unclear whether the apparent increase in nestin expression would translate into functional improvements over a longer period or if multiple doses of AD-MSCs would sustain or further improve these results. A longitudinal study exploring these variables could provide more comprehensive insights into the therapeutic potential of AD-MSCs.[32,33]

This is important to compare the efficacy of AD-MSCs with other types of stem cells in Alzheimer's treatment as well. While neural stem cells and bone marrow-derived mesenchymal stem cells have shown some promise, AD-MSCs have the advantages of easier accessibility, lower immunogenicity, and higher proliferation rates, making them an increasingly appealing option for researchers.[34,35] The use of a single dosage and a short-term study model may not entirely capture the long-term effects and sustainability of AD-MSCs in modulating nestin expression and contributing to neurogenesis. This opens avenues for future research to explore these dimensions further.[36] Additionally, it would be worth investigating the molecular pathways through which AD-MSCs influence nestin expression. Understanding these pathways could lead to more targeted therapeutic strategies for AD, maximizing of the neurogenic potential of AD-MSCs.[36,37] The findings of the current study pave the way for future in-depth studies to explore the long-term effects, safety profiles, and precise molecular mechanisms through which AD-MSCs exert their therapeutic effects. Ongoing experiments are focused on exploring the interaction of AD-MSCs with other cellular pathways impacted in AD, moving closer to a comprehensive, stem cell-based therapeutic strategy for this devastating neurodegenerative disease.[38]

Conclusion

In conclusion, this study significantly advances the field of AD treatment by highlighting the therapeutic potential of AD-MSC-like cells in restoring nestin gene expression, a critical marker associated with neural plasticity and neurogenesis. AD-MSC-like cells also show promise in mitigating the amyloid plaque burden, and by extension, could be beneficial in slowing the progression of AD.

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Authors Contributions

A and HA were involved in concepting and planning the research, A and GR performed the data acquisition/collection, A, GR and A calculated the experimental data and performed the analysis, A drafted the manuscript and designed the figures, HA and A aided in interpreting the results. All authors took parts in giving critical revision of the manuscript.

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