


Adipose Tissue-Derived Mesenchymal Stem Cells Alter Metabolites of Brain Cholesterol Homeostasis in An Alzheimer's Model

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Abstract

Objective: Disruption of cholesterol homeostasis in Alzheimer's disease (AD) plays a crucial role in disease pathogenesis, making it a potential therapeutic target. Mesenchymal stem cells (MSCs) show promise in treating cognitive impairment and provide a novel therapeutic approach. This study aims to investigate the effects of MSCs on specific metabolites associated with brain cholesterol homeostasis in an AD rat model.

Materials and Methods: In this experimental study, animals were divided into three groups: control, AD, and AD+MSCs. AD was induced using amyloid beta (A β) and confirmed through the Morris water maze (MWM) behavioural test and Congo red staining. MSCs were extracted, characterised via flow cytometry, subjected to osteoblast and adipose differentiation, and injected intraventricularly. The cholesterol metabolite levels were measured using gas chromatography-mass spectrometry (GC)-MS and compared among the groups.

Results: Treatment with MSCs significantly improved memory function in the AD+MSCs group compared to the AD group and the number of beta-amyloid plaques decreased according to histological assessment. Disturbances in the brain cholesterol metabolites that included desmosterol, 7-dehydrocholesterol, 24S-hydroxycholesterol, 27-hydroxycholesterol and cholesterol were observed in the AD group compared to the control group. Treatment with MSCs resulted in significant alterations in the levels of these metabolites.

Conclusion: The findings indicate that MSC therapy has the potential to improve AD by modulating brain cholesterol homeostasis and promoting the differentiation of stem cells into nerve cells. The results emphasize the importance of investigating the role of cholesterol metabolites in the context of MSC therapy to gain deeper insights into underlying mechanisms of the therapeutic efficacy of MSCs in AD.

Keywords: Alzheimer's Disease, Desmosterol, 7-Dehydrocholesterol, 24-Hydroxycholesterol, 27-Hydroxycholesterol

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Introduction

Alzheimer's disease (AD) is a devastating disease of the nervous system (1), and a disruption in cholesterol homeostasis plays a role in its development. Although the brain comprises approximately 2% of the body's total weight, it contains approximately 25% of the body's cholesterol. This fact highlights the crucial role of cholesterol in the brain. Brain cholesterol is essential for numerous functions, including synapse formation and nerve signal transmission (2). Cholesterol homeostasis

is critical for the normal functioning of nerve cells (3), and its disturbance is considered a risk factor for the development of AD. Therefore, brain cholesterol homeostasis is preserved via a precise regulatory process that involves cholesterol biosynthesis and the elimination of its metabolites (4).

Brain tissue cholesterol is produced in neurons and astrocytes by two separate pathways, namely Kandutsch-Russell and Bloch (Fig.1). Defects in either of these

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pathways cause serious damage to the nervous system (3). The precursors of cholesterol in the Kandutsch-Russell and Bloch pathways are 7-dehydrocholesterol and desmosterol, respectively, which are synthesized by the enzymes 7-dehydrocholesterol reductase (DHCR7) and 24-dehydrocholesterol reductase (DHCR24) (5). Excess cholesterol in neurons and astrocytes is converted into more polar metabolites known as oxysterols, which are excreted into the circulation through the blood-brain barrier (BBB). Oxysterols are key signalling compounds for brain function, and it is believed that cholesterol may perform part of its function in the brain through these metabolites (6). One of these compounds is 24-hydroxycholesterol, which is generated in neurons from excess cholesterol by the enzyme 24-hydroxylase (CYP46A1) (7). By binding to liver X (LX) receptors, 24-hydroxycholesterol induces the expressions of *APOE* and *ABCA1* genes, thereby regulating the influx of cholesterol from astrocytes to neurons. Another important oxysterol is 27-hydroxycholesterol, which is produced by the enzyme 27-hydroxylase (*CYP27A1*), and can activate LX receptors (2). It seems that the pathway of cholesterol homeostasis changes during the formation of amyloid beta ($A\beta$) plaques and

other mechanisms of AD, and it is significant in the development of this disease. This pathway might also play a role in AD pathogenesis and can be a consequence of neurodegeneration (8). Thus, considering the importance of maintaining cholesterol homeostasis, this pathway can be considered a therapeutic target for AD treatment.

To date, a definitive treatment for AD remains elusive, and available medications only serve to slow disease progression and alleviate symptoms (9). In recent years, stem cell therapy has emerged as a promising research methodology. Stem cells are believed to exert their therapeutic effects by migrating to the site of injury where they differentiate into specific cell types and establish vital connections and synapses (10). There is a growing interest in utilising stem cells to treat central nervous system disorders. The significance of cholesterol homeostasis, particularly specific cholesterol metabolites like 24-hydroxycholesterol, plays a crucial role in memory and learning. Therefore, the objective of this study is to investigate the impact of adipose tissue-derived mesenchymal stem cells (MSCs) on the levels of various metabolites involved in the cholesterol homeostasis pathway in the brain tissue of an animal model of AD.

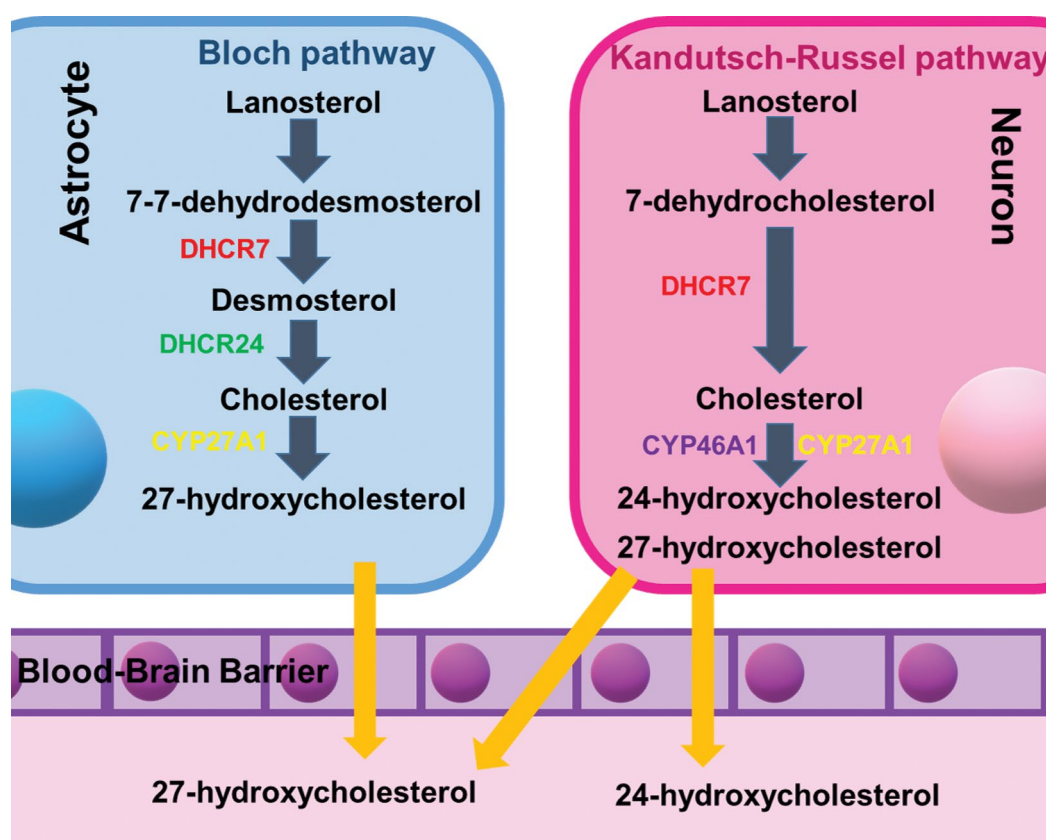


Fig.1: Pathway of cholesterol synthesis and oxysterol formation in neurons and astrocytes. DHCR7; 7-dehydrocholesterol reductase and DHCR24: 24-dehydrocholesterol reductase.

Materials and Methods

Animals

In this experimental study, 24 male Wistar rats that weighed 250-300 g were acquired from the Animal Centre of Ahvaz Jundishapur University of Medical Sciences (Ahvaz, Iran). The animals were kept at 21-25°C with controlled humidity and a 12/12 hour light/dark cycle with lights on at 7:00 AM as well as unlimited access to food and water. All animal procedures were carried out according to the guidelines of the Ethics Committee and the Centre of Research on Laboratory Animals at Ahvaz University of Medical Sciences, Ahvaz, Iran (IR.AJUMS.ABHC.REC.1400.106). The animals were divided into three groups: i. The control group (n=8) received a single bilateral intrahippocampal (I.H.P.) injection of 5 µl sterile phosphate-buffered saline (PBS), ii. The AD group (n=8) received 5 µM/µL/rat Aβ bilateral I.H.P., and iii. The AD+MSCs group (n=8) received a single bilateral intraventricular injection of 1×10⁶ MSCs, 30 days after the stereotaxic injection of Aβ.

Preparation of synthetic amyloid beta oligomers

The Aβ 1-42 peptide was activated with PBS. Briefly, Aβ1-42 was dissolved in PBS and stored at -70°C. For the aggregation protocol, sterile PBS was added to bring the peptide to a final concentration of 1 µg/µL, and then incubated for five days at 37°C.

Stereotaxic surgery

The animals were first anaesthetised by an intraperitoneal injection of ketamine/xylazine (90:10 mg/kg), and their heads were shaved and fixed in a stereotaxic apparatus. For the control group, we used a 10-µL Hamilton syringe to inject 5 µl PBS and bilateral injections of 5 µl Aβ (2.5 µL per side at a rate of about 1 µL/minutes) into the hippocampus of the CA1 region at the coordinates of AP=-4.3, ML= ± 2.4, and DV=-2.6 mm (Paxinos and Watson stereotaxic rat brain atlas) (11). For the AD+MSCs group, the animals were first treated with Aβ to induce Alzheimer's, and then 1×10⁶ MSCs (12) dissolved in PBS was injected intraventricularly at the coordinates of AP=-1.2, ML= ± 2, and DV=-3.8 (11).

Isolation of adipose tissue-derived mesenchymal stem cells

The animals were first anaesthetised with ketamine/xylazine, and the epididymal fat tissues of the animals were isolated. All of the isolation stages were performed under sterile conditions. The minced tissues were transferred to sterile falcon tubes that contained 1% penicillin/streptomycin (pen/strep) dissolved in PBS and subsequently washed to eliminate red blood cells and connective tissue. Next, 1 mg/mL of collagenase - was added, and the samples were placed in a shaking incubator at 37°C for 40 minutes. Afterwards, they were centrifuged for 15 minutes at 1500 rpm and the final sediment was incubated with Dulbecco's modified eagle medium, 10%

FBS, 1% pen/strep, and 1% amphotericin in a culture flask at 37°C, 5% CO₂, and 95% humidity. The medium was changed every three days (13).

Cell characterisation

Flow cytometry

Passage-3 cells were assessed by flow cytometry. For this purpose, we used conjugated antibodies anti-mouse-CD44-FITC and anti-mouse-CD90-FITC (stem cell markers), and anti-mouse-CD34-RPE and anti-mouse-CD45-RPE (hematopoietic markers) with a negative control (Denmark, Glostrup, Corporation Dako). The data were analysed by FlowJo software (version 7.6.1, FlowJo, LLC, Ashland, Oregon, United States).

Osteoblast and adipose differentiation

Passage-3 mesenchymal cells were grown in osteogenic differentiation medium (10 mM Beta glycerol phosphate, 7-10 mM dexamethasone, 10% FBS, 50 µg/mL ascorbic acid bi-phosphate, 1% pen/strep-amphotericin B) and adipogenic differentiation medium [66 nM insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.2 mM indomethacin, 7-10 mM dexamethasone, 10% FBS, and 1% pen/strep-amphotericin B]. The cells were incubated at 37°C and 5% CO₂ for 21 days and the medium was replaced every two days. Alizarin red and oil red O staining were conducted to confirm differentiation into osteoblasts and adipocytes, respectively (13).

Morris water maze behavioural assessment

This test is used to assess memory and spatial learning in rats. In the morris water maze (MWM) test, a round metal tank with a black wall that is 120-200 cm in diameter and 70 cm in height is filled with water to a depth of 60 cm with an optimal water temperature of 25 ± 2°C. An invisible platform with a diameter of 12 cm is positioned at a distance of 1 to 5 cm below the water surface in the centre of one of the four quadrants (northeast, southeast, northwest, or southwest). The movement and behaviour of the animals is tracked and controlled by an infrared camera placed at a height of 2 m above the central area of the tank. The animals were examined for five consecutive days. This experiment was conducted 30 days after the Aβ injection for the AD group and at 30, 60, and 90 days after the MSC injection for the AD+MSC group. During the first four days, the animal's learning process was measured based on the time spent and the distance travelled to find the hidden platform. Accordingly, each animal was randomly placed in a quadrant for 60 seconds to find the hidden escape platform and then given a 30 second rest until the next trial. On the fifth day, a probe phase was performed in order to assess the animal's memory performance. The hidden platform was removed and the percentage of time spent in the goal quadrant during a single trial, the location of the platform, and the distance moved in this quadrant were determined as the standard measure of memory (14).

Extraction of brain tissue

Each animal was anaesthetised using a combination of ketamine/xylazine, and then pinched to assess the level of anaesthesia. Next, the rat was perfused with PBS, decapitated by a specialized guillotine, and then the brain was extracted. Part of the tissue was fixed with 10% formaldehyde to prepare a slide and stained with Congo red dye to confirm formation of the A β plaques. The remainder of the tissue was collected in cryotubes and stored at -70°C.

Congo red staining

In order to detect the amyloid deposits, the prepared brain sections were subjected to Congo red staining according to the manufacturer's protocol (Commercial kit, Fartest). A light transmitter was used to detect the presence of amyloid plaques, which were observed as a pink to red colour.

Measurement of cholesterol metabolites

First, the lipid content of the brain tissue was extracted. Then, the cholesterol metabolites desmosterol, 7-dehydrocholesterol, 24-hydroxycholesterol, and 27-hydroxycholesterol were measured using a chromatograph (model 6890) coupled with a mass spectrometer (model 5973N, Agilent, America) and an HP-5MS capillary column with a stationary phase of 5% methylphenylsiloxane (length: 30 m, inner diameter: 0.25 mm, thickness of the stationary layer: 0.25 μ m) and ionisation energy of 70 eV. Briefly, 25 mg of brain tissue was homogenized with 88% methanol that contained 0.01% butylated hydroxytoluene at a temperature of 4°C using a homogenizer (Heidolph, Germany), then 100 μ L of methanol and 250 μ L of 1 M NaOH were added and the mixture was incubated for 16 hours at room temperature in the absence of light for hydrolysis. The samples were acidified with 1 M formic acid, followed by the addition of distilled water. In order to extract the solid phase, solid phase extraction columns (GL Sciences, USA) were prepared with methanol and 40 mM formic acid. The sample that contained sterol and oxysterol was removed from the column by hexane and hexane/methyl tert-butyl ether and dried at 37°C under N₂ gas flow, and then prepared in toluene for mass spectrometry (MS) analysis.

Statistical analysis

The results of all the analyses are expressed as mean \pm standard error of the mean (SEM) for continuous variables. Differences between the three groups were determined using a one-way ANOVA test in GraphPad Prism software, version 6 (Dotmatics, California). The level of significance was considered to be $P < 0.05$.

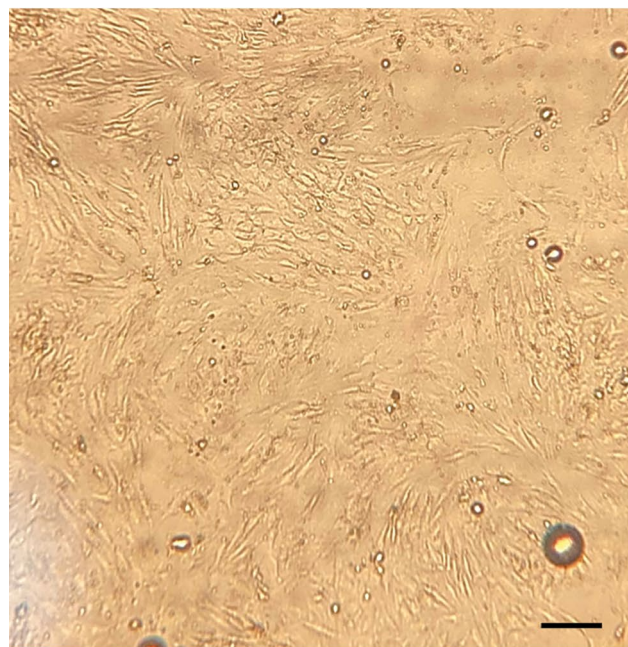
Results

Characteristics of mesenchymal stem cells

In the initial culture, adipose-derived MSCs with

a fibroblast-like or spindle-shaped appearance with distinct nuclei were grown, which were observed by light microscopy (Fig.2).

A



B

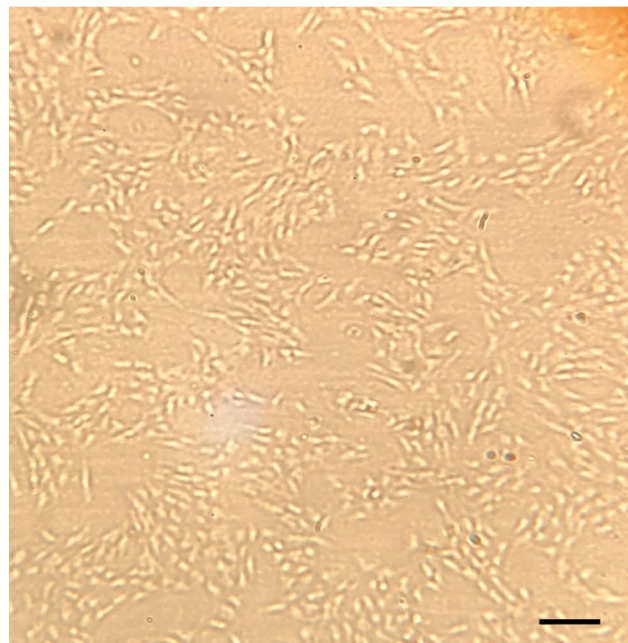


Fig.2: Isolation of MSCs from adipose tissue. MSCs culture. **A.** Primary stage. **B.** First passage (scale bar: 100 μ m). MSC; Mesenchymal stem cells.

Flow cytometry

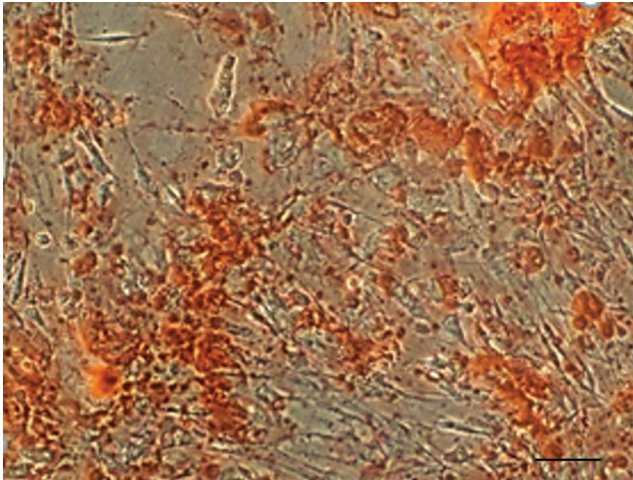
MSCs extracted from adipose tissue expressed CD44 (98.98%) and CD90 (94.34%), which are stem markers for these cells. The hematopoietic markers, CD34 (0.13%) and CD45 (0.43%), had very low expression (Fig.S1, See

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Differentiation of mesenchymal stem cells into osteocytes and adipocytes

Figure 3 shows that the multipotent adipose MSCs differentiated into osteocytes and adipocytes.

A



B

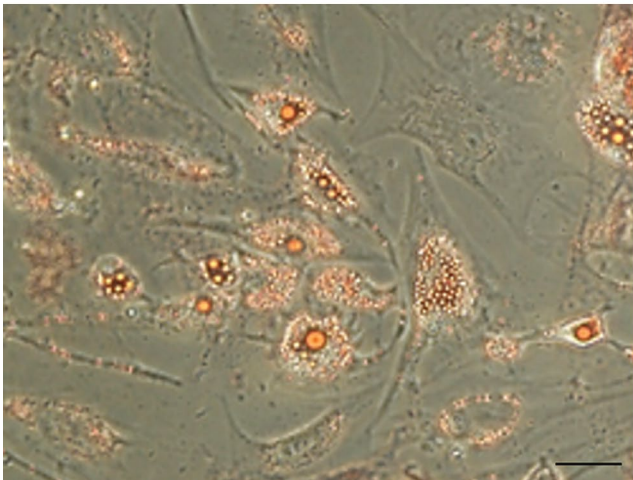


Fig.3: Differentiation of adipose mesenchymal stem cells (MSCs) into adipocytes and osteoblasts. **A.** Osteoblasts stained with alizarin red (scale bar: 100 μ m). **B.** Adipocytes stained with oil red O (scale bar: 50 μ m).

Spatial learning and memory in the Morris water maze test

Spatial memory of animals from all three groups was examined four weeks after the A β injection. In the probe trial, the AD group had a significantly less percentage of time spent in the target quadrant than the control and AD+MSC groups ($P=0.0001$), which indicated impaired spatial learning and memory. This scale showed a significant increase in the AD+MSC group compared to the AD group ($P=0.0001$, Fig.4). There was no difference in swimming speed between all of the studied groups.

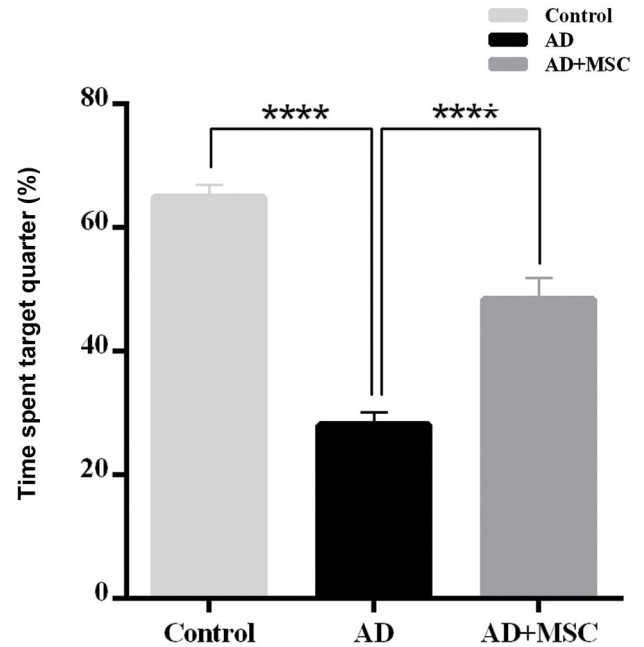


Fig.4: MWM behavioural test diagram shows the effect of A β and MSCs on spatial memory of animals from all three groups. The AD group shows a significant reduction in the percentage time spent in the target quadrant in the probe trial ($P=0.0001$). There is a significant increase in the AD+MSC group compared to the AD group ($P=0.0001$). MWM; Morris water maze, A β ; Amyloid beta, MSC; Mesenchymal stem cell, AD; Alzheimer's disease, and ****; $P<0.0001$.

Histology

Sections from the animals' brains were stained with Congo red to detect the A β plaques. This dye has a high affinity for binding to insoluble A β plaques. In the control group (Fig.5A), no amyloid plaque was observed, while, Figure 5B shows the presence of these plaques in the brain slices of the animals 30 days after the A β injection. The number of these plaques in the AD+MSC group was significantly reduced 90 days after administration of the MSCs (Fig.5C).

Gas chromatography-mass spectrometry

Desmosterol, which is the precursor of cholesterol synthesis in astrocytes, was significantly increased in the AD group compared to the control group ($P=0.0001$), while the level of this metabolite in the AD+MSC group showed a significant reduction compared to the AD group ($P=0.0001$, Fig.6A). In this regard, the measurement of 7-dehydrocholesterol, which is the precursor of cholesterol in the Kandutsch-Russell pathway in neurons, was similar to the results observed with desmosterol (Fig.6B). A significant increase in cholesterol was observed in the AD group compared to both the control and AD+MSC groups ($P=0.0001$, Fig.6C). Metabolite 24-hydroxycholesterol, a by-product of the Bloch pathway in astrocytes, was significantly decreased in the AD group compared

to the control group ($P=0.0001$), but significantly increased in the AD+MSC group compared to the AD group ($P=0.0001$, Fig.6D). The observed results

with 27-hydroxycholesterol, a by-product of the Kandutsch-Russell pathway, were similar to those of 24-hydroxycholesterol (Fig.6E).

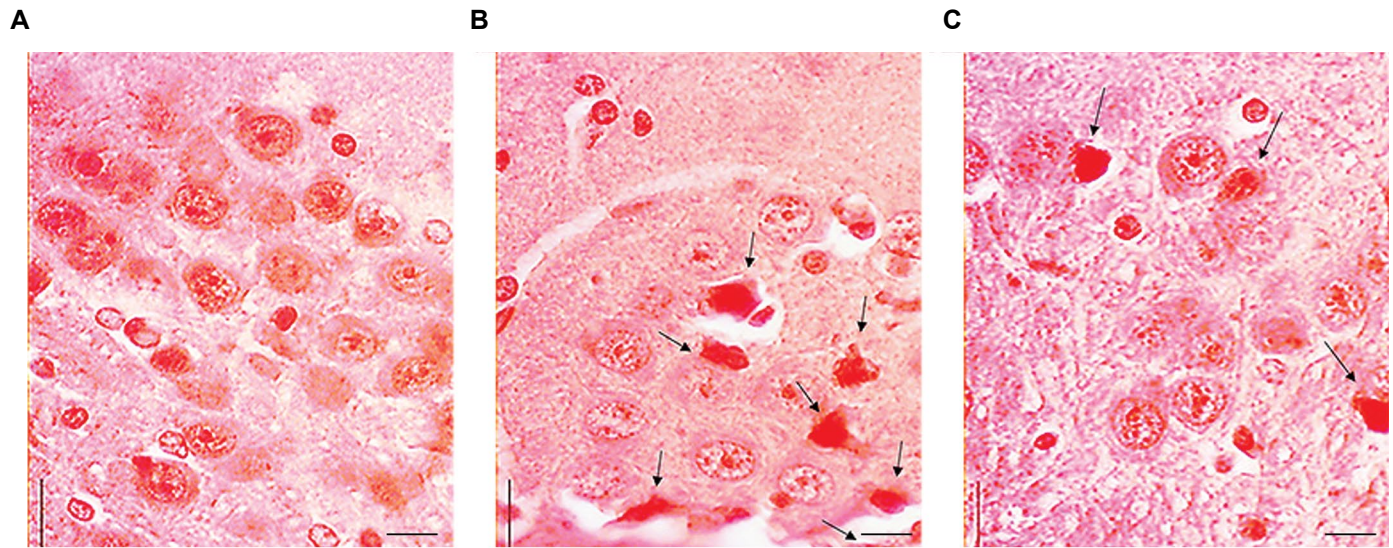


Fig.5: Congo red staining of the brain tissues. **A.** Control group, **B.** AD group, and **C.** AD+MSC group (scale bar: 20.0 μm). Black arrow shows $\text{A}\beta$. AD; Alzheimer's disease, MSCs; Mesenchymal stem cells, and $\text{A}\beta$; Amyloid beta.

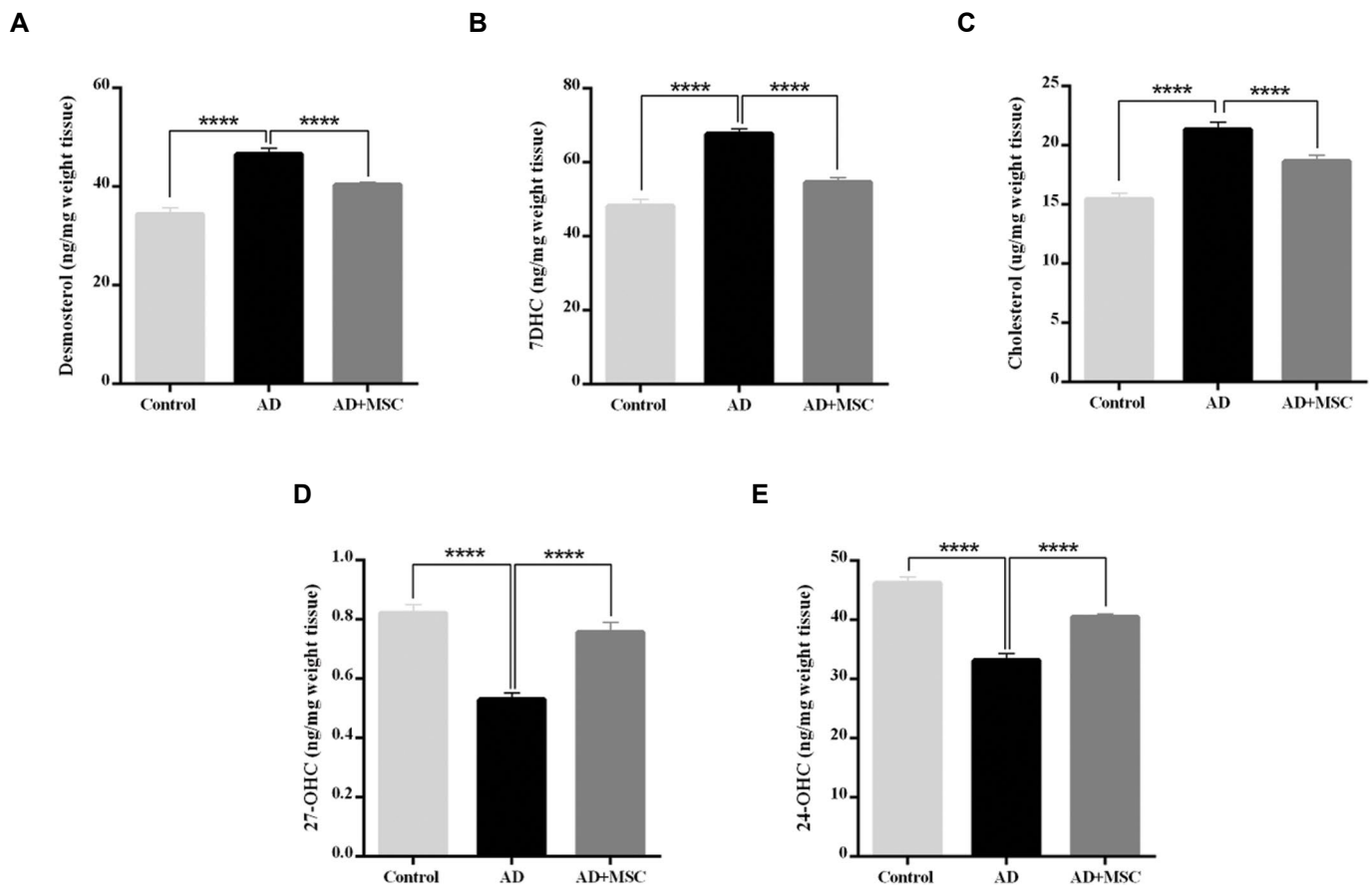


Fig.6: Measurement of cholesterol homeostasis pathway metabolites by GC-MS in the control, AD, and AD+MSC groups. **A.** Desmosterol, **B.** 7-dehydrocholesterol, **C.** Cholesterol, **D.** 24-hydroxycholesterol, and **E.** 27-hydroxycholesterol. AD; Alzheimer's disease, MSCs; Mesenchymal stem cells, GC-MS; Gas chromatography-mass spectrometry, and ****; $P \leq 0.0001$.

Discussion

AD is the most prevalent dementia in the elderly (15), and its main features are formation of amyloid plaques outside the cell and phosphorylated tau proteins inside the cell (1). The results of the current study indicate that an injection of MSCs into the Alzheimer's animal model leads to the improvement in memory, a change in the amount of A β plaques in the brains of the group that received the MSCs compared to the AD group, and significant modification in cholesterol homeostasis in the AD+MSC group compared to the AD group.

According to numerous studies on the relationship between cholesterol homeostasis and AD, disruption in the regulation of cholesterol homeostasis might reflect the basic features of AD pathogenesis, and therefore the cholesterol content of brain tissue in the AD group differs from the control group (8). In this regard, the results of our study also revealed a 38% rise in the cholesterol content in the tissue from the AD group. Potential reasons for the cholesterol increase in Alzheimer's brain tissue include the destruction of neurons, the breakdown of the plasma membrane, and the deterioration of myelin (16). Evidence has shown that the destruction of myelin leads to the release of cholesterol into the cerebrospinal fluid and extracellular space, which ultimately results in the death of nerve cells (17). On the other hand, *in vitro* studies have demonstrated that this excess cholesterol also leads to an increase in the production of A β in the brain tissue, and subsequently, these A β plaques cause extensive neuronal loss, the breakdown of myelin, and the release of excess cholesterol in the extracellular space (18). Following the injection of MSCs, the level of cholesterol in the AD+MSC group significantly decreased by 12.6% compared to the AD group. The possible mechanism is the differentiation of multipotent MSCs into various types of nerve cells, including glial cells and neurons, which can help regulate cholesterol levels by secreting a variety of beneficial cytokines and neurotrophic factors (19).

Cholesterol is synthesised *de novo* in astrocytes and neurons from a series of precursors and converted into a series of more polar metabolites to exit the brain through the BBB (20). Cholesterol is produced in astrocytes through the Bloch pathway from the 27-carbon precursor desmosterol by the enzyme DHCR24. In the current study, the level of desmosterol precursor in the AD group significantly increased by 35% compared to the control group, which is likely due to the disruption or decrease in the expression of the DHCR24 enzyme caused by the pathogenesis of AD (21).

Contrary to our results, Mohhammad et al. (4) showed a reduction in the level of desmosterol in patients compared to a control group, which prompted the speculation that other factors might also play a role in regulating desmosterol levels. One of these factors is the hormone progesterone, which inhibits cholesterol synthesis in the stage between lanosterol and cholesterol (22). However, it is hypothesised that the decrease in the level of this metabolite correlates

with AD progression, which is marked by the loss of more neurons (23). Similarly, 7-dehydrocholesterol is a precursor of cholesterol synthesis in the Kandutsch-Russell pathway, and according to the results, this metabolite showed a significant increase of 41% in the AD group compared to the control group. This increase is probably due to the decrease in the gene expression of the 7-dehydrocholesterol reductase enzyme, which converts 7-dehydrocholesterol to cholesterol (24). Disturbances in these two pathways may lead to the replacement of cholesterol by its precursors in the brain, which causes serious disorders in the nervous system (25). Similar to cholesterol, these precursors can bind to the SREBP cleavage-activating protein (SCAP) and alter its conformation, which in turn changes the level of cholesterol synthesis (2). This finding might justify the increase in cholesterol levels in the AD group compared to the control group. In this study, the injection of stem cells in the AD+MSC group significantly decreased the level of desmosterol and 7-dehydrocholesterol compared to the AD group, which supported the other findings of this study and indicated the positive effect of these cells on various aspects of this disease.

No pathway exists to destroy cholesterol in the brain; therefore, excess cholesterol is converted into 24-hydroxycholesterol and 27-hydroxycholesterol by the enzymes CYP46A1 and CYP27A1, respectively, and enters the bloodstream through the BBB (26). CYP46A1 activity and 24-hydroxycholesterol are not only necessary for memory processing but can also reduce A β levels (27). Research has shown that the presence of amyloid plaques and nerve cell destruction appeared to impair the activity of cholesterol-hydroxylating enzymes, and resulted in a 30% reduction in the level of metabolites in the brain tissue of Alzheimer's patients compared to a control group (28). In the AD+MSC group, the level of these byproducts significantly increased by 25% compared to the AD group after the injection of the MSCs. This finding is in line with the role of these metabolites and the enzymes that produce them in improving memory and reducing the level of A β . The results of the present study showed that not only the number of plaques in this group reduced, but memory and learning also improved significantly.

Numerous studies have shown that MSCs can differentiate into various types of nerve cells and can be a suitable option for cell therapy purposes in neurodegenerative diseases (29). The paracrine effects of MSCs, including the production of growth factors and anti-inflammatory cytokines, lead to nerve regeneration and myelination. MSCs likely exert phagocytic effects on abnormal A β plaques as well as anti-inflammatory effects in the AD brain via microglia, prevention of neuronal death, and enhancement of neuronal differentiation (12). Since the disorders of the cholesterol homeostasis pathway and the reduction or lack of activity of the enzymes in this pathway are more related to the loss of nerve cells, it is possible that replacing nerve cells with differentiated MSCs will cause a slight improvement in these defects,

and result in enhanced memory and a reduction in the amount of A β plaques in the animal's brain tissues.

Conclusion

Our research findings provide compelling evidence that MSC therapy has the potential benefit for improving various aspects of AD neuropathogenesis. We observed positive outcomes, including the recovery of memory function, reduced numbers of A β plaques, and modulation of cholesterol homeostasis in an animal model of AD. These results highlight the promising prospects of MSC therapy as a viable therapeutic approach for AD. Furthermore, targeting cholesterol homeostasis through MSC-based interventions may open a novel way for AD treatment. Further investigations are necessary to comprehensively understand the underlying mechanisms and optimise the therapeutic potential of MSCs in the context of AD.

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Authors' Contributions

M.Ch., A.Kh., Z.N., Y.F.; Conceptualisation, Methodology, and Software. M.K.D., S.P.P.; Data curation, Conducting experiments, Writing - Original draft preparation, and Supervision. Sh.A., M.A.; Visualisation and Investigation. A.R.; Software and Validation. M.Ch., M.K.D.: Writing- Reviewing and Editing. All authors read and approved the final manuscript.

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