Exploring the molecular mechanisms of MSC-derived exosomes in Alzheimer's disease: Autophagy, insulin and the PI3K/Akt/mTOR signaling pathway

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ARTICLE INFO

ABSTRACT

Alzheimer's disease (AD) is a devastating neurological condition characterized by cognitive decline, motor co-ordination impairment, and amyloid plaque accumulation. The underlying molecular mechanisms involve oxidative stress, inflammation, and neuronal degeneration. This study aimed to investigate the therapeutic

Autophagy
Neuroinflammation

effects of mesenchymal stem cell-derived exosomes (MSC-exos) on AD and explore the molecular pathways involved, including the PI3K/Akt/mTOR axis, autophagy, and neuroinflammation. To assess the potential of MSC-exos for the treatment of AD, rats were treated with AlCl$_3$ (17 mg/kg/once/day) for 8 weeks, followed by the administration of an autophagy activator (rapamycin), or MSC-exos with or without an autophagy inhibitor (3-methyladenin; 3-MA+ chloroquine) for 4 weeks. Memory impairment was tested, and brain tissues were collected for gene expression analyses, western blotting, histological studies, immunohistochemistry, and transmission electron microscopy. Remarkably, the administration of MSC-exos improved memory performance in AD rats and reduced the accumulation of amyloid-beta (Aβ) plaques and tau phosphorylation. Furthermore, MSC-exos promoted neurogenesis, enhanced synaptic function, and mitigated astrogliosis in AD brain tissues. These beneficial effects were associated with the modulation of autophagy and the PI3K/Akt/mTOR signalling pathway, as well as the inhibition of neuroinflammation. Additionally, MSC-exos were found to regulate specific microRNAs, including miRNA-21, miRNA-155, miRNA-17–5p, and miRNA-126–3p, further supporting their therapeutic potential. Histopathological and bioinformatic analyses confirmed these findings. This study provides compelling evidence that MSC-exos hold promise as a potential therapeutic approach for AD. By modulating the PI3K/Akt/mTOR axis, autophagy, and neuroinflammation, MSC-exos have the potential to improve memory, reduce Aβ accumulation, enhance neurogenesis, and mitigate astrogliosis. These findings shed light on the therapeutic potential of MSC-exos and highlight their role in combating AD.

1. Introduction

Alzheimer's disease (AD) is the primary cause of dementia in the aged population worldwide. AD is an irreversible, progressive neurological condition. According to estimates, 35.6 million people were diagnosed with dementia worldwide in 2010 [1]. By 2050, this number is predicted to double every 20 years. The cognitive impairment and memory loss that hinder one's capacity to carry out daily tasks on one's own are features of AD. With the increasing incidence of AD worldwide, the expense of caring for these patients is growing, placing a significant strain on people, families, and society [2].

Efforts to investigate the aetiology of AD have increased, but the causes remain unknown, and there are currently no viable treatments available [3]. Senile plaques made of accumulating b-amyloid peptide (Aβ) and neurofibrillary tangles (NFTs) containing highly phosphorylated tau are the pathological hallmarks of Alzheimer's disease (AD) [4]. Aβ-mediated effects have been linked to autophagy, and disruption of autophagy is critical in neurodegenerative illnesses [5], as insufficient removal of toxic and abnormal protein aggregates leads to cellular stress and eventually failure and death. Consequently, stimulating autophagy is a suggested tactic for assisting neurons in eliminating aberrant protein aggregates. The autophagic process is regulated by the mammalian target of rapamycin (mTOR) kinase, which is influenced by cellular stresses, growth factors, and hunger [6]. The PI3K/AKT survival pathway, which is located upstream of mTOR, regulates mTOR activity and is altered in neurodegenerative illnesses such as Parkinson's disease and Alzheimer's disease. However, a thorough analysis of the intricate relationship between the PI3K/AKT/mTOR pathway and the autophagic process is necessary [7].

Several preventive and therapeutic strategies for AD have been proposed, one of which involves the use of mesenchymal stem cells (MSCs). MSCs have recently garnered significant interest as possible cell-based therapeutic tools because of their ability to migrate and mediate damage repair [8]. Multiple disease models, including atopic dermatitis, myocardial infarction, traumatic brain injury, and diabetic nephropathy, have demonstrated the immunomodulatory function, migratory capability, and regeneration potential of MSCs [9]'[10]'[11]'[12]. By secreting neurotrophins and angiogenesis-regulating factors, MSCs promote neurological recovery and neoangiogenesis. In fact, stem cells have been used to treat multiple sclerosis, spinal cord injuries, stroke, brain tumours, and peripheral nerve damage [13]. Nevertheless, a growing body of research indicates that MSC biodistribution and persistence in target organs are uncommon and that the therapeutic benefit likely results from paracrine activity [14]'[15]'[16]. According to proteomic and electron microscopy research, exosomes are part of the paracrine component of MSCs [17].

Exosomes, also known as small extracellular vesicles (sEVs), are released by a range of cell types and are thought to be a subtype of extracellular macrovesicles with a diameter of 30–150 nm [18] [19]. Exosomes are encased in a lipid bilayer membrane. Extracellular vesicles were once thought to be cellular trash when they were first proposed to exist in mammalian tissues or bodily fluids in the 1960s [20]'[21]'[22]. Because exosomes can deliver biologically active cargo, including proteins, lipids, and nucleic acids, to recipient cells in both healthy and pathological settings, they are now understood to be mediators of intercellular communication ([23–25]). Currently, exosomes are being investigated for use as immunosuppressants, vaccines, and stimulators of differentiation and repair processes [26].

Exosomes have been implicated in many illnesses of the central nervous system (CNS) as important mediators of cell-to-cell and distant communication. This may occur through the transfer of exosome contents, which include lipids and nucleic acids, to the target cells. Exosomes have been shown to store and disperse proteins associated with AD, including Tau and Aβ [27], [28]. Because brain-derived exosomes can pass through the blood—brain barrier, it is possible to isolate them from the blood and use them to study the pathophysiology of AD [29]. Recently, Chen et al. reported that exosomes generated from mesenchymal stem cells restored synaptic plasticity and neural memory in AD transgenic mice and decreased Aβ levels in an in vitro model of AD [30]. However, the underlying chemical pathways are not yet entirely understood.

Here, we sought to summarize the function of exosomes in the management of AD. Furthermore, we assessed the therapeutic mechanisms of MSC-exos in AD, paying particular attention to the functions of autophagy regulation and the PI3K/AKT/GSK-3β pathway.

2. Materials and methods

2.1. Chemicals, reagents, and kits

Rapamycin (Gene Operation, Ann Arbor, MI, USA) was dissolved in DMSO to a concentration of 100 mg/ml, further diluted in 5 % PEG (Sigma P3265)-400/5 % Tween-80 (Sigma P1754) to a final concentration of 1.2 mg/ml, sterile filtered, and kept at 80 °C [31]. AlCl$_3$ (MW=133.34) was purchased from Sigma—Aldrich Co. (Munich, Germany). 3-MA was dissolved in distilled water at 50 ºC (NoM9281, Sigma—Aldrich, USA). Chloroquine diphosphate (5428–61–5, Sigma—Aldrich, USA) was dissolved in distilled water. Primary antibodies against AMPK (sc-74461, Santa Cruz, USA), mTOR (sc-517464, Santa Cruz, USA), p-GSK-3β (sc-373800, Santa Cruz, USA), p-PI3K (E-AB-20966, ELABSCIENCE, China), PI3K (E-AB-64202, ELABSCIENCE, China), p-AKT1 (E-AB-20804, ELABSCIENCE, China), AKT1 (E-AB-15441, ELABSCIENCE, China), p-Tau (E-AB-20990, ELABSCIENCE, China), Tau (E-AB-33036, ELABSCIENCE, China), NF-κB-p65

(abx012874, Abbexa, Germany), iNOS (sc-136977, Santa Cruz, USA), β-actin (E-AB-20031, ELABSCIENCE, China), and synaptophysin (ab8049, Abcam, UK) were used. Anti-GFAP (ab7260, Abcam, UK) and anti-CD63 (PA5–92370, Thermo Fisher, USA) antibodies were used. ELISA kits for P62 (SG-22399) and LC3BII (SG-24264) were purchased from SinoGeneClon Biotech (China).

2.2. Preparation of MSC-exos

2.2.1. Culture of MSCs
MSC-exos were prepared using conditioned media from bone marrow-derived MSCs (BM-MSCs). Rat BM-MSCs were obtained from the Benha University Faculty of Medicine Central Laboratory [32]' [33]. The cells were grown overnight in Dulbecco's Modified Eagle Medium (DMEM), which contains 0.5 % human serum albumin (HSA) but not foetal bovine serum (FBS) (Sigma—Aldrich, St. Louis, MO, USA). This action was taken to stop cell death and decrease the amount of released cell debris and apoptotic bodies in conditioned medium [34]. Trypan blue exclusion was used to determine the viability of the cells cultivated overnight, which exhibited greater than 99 % viability.

2.2.2. Isolation of MSC-exos
Exosomes were isolated from MSCs after the first, second, and third passages in DMEM without FBS. Exosomes were isolated by ultracentrifugation of the conditioned media at 4 °C for an hour at $100,000 \times g$ with a SW41 swing rotor after first being centrifuged for 20 minutes at $2000 \times g$ to remove debris (Beckman Coulter, Fullerton, CA, USA). Serum-free M199 solution containing 25 mM 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) (pH = 7.4) (Sigma—Aldrich, St. Louis, MO, USA) was used to wash the separated exosomes before ultracentrifugation. Using magnetic stirring and disc membranes from the marketplace with 0.1 mm pore diameters, 100 ml of the finished exosomes were microfiltered (VVLP; Millipore; Bedford, MA, USA). A Millipore Amicon 50-ml stirred cell apparatus was used to house the disc membranes. Finally, the exosomes were collected, analysed, and stored at −80 °C until further use [35].

2.2.3. Electron microscopy characterization of MSC-exos
The isolated exosomes were fixed after being incubated in 2.5 % glutaraldehyde in HAS for 2 hours. The exosomes were prepared after washing, ultracentrifugation, and suspension in HSA (100 l). Twenty microlitres of exosomes were deposited on Formvar/carbon-coated grids and negatively stained with 3 % aqueous phosphotungstic acid for 1 min before being examined under a transmission electron microscope (Hitachi H-7650, Hitachi, Tokyo, Japan) [36].

2.2.4. Flow cytometry characterization of MSC-exos
Both CD105 (a marker for MSCs) and CD63 (an exosome marker) were used in the cytofluorimetric study. Exosomes were cleaned and then resuspended in phosphate-buffered saline (PBS) supplemented with 3 % foetal bovine serum (FBS) and saturating concentrations (1:100) of the anti-CD63 (ab18235) and anti-CD105 (BD Pharmingen) fluorescein isothiocyanate-conjugated anti-human monoclonal antibodies. Forwards and side scatter analyses were used to analyse the samples (Becton-Dickinson, Canada).

2.2.5. Protein content of MSC-exos
The protein content of the exosomes was measured using the Bradford method (Bio-Rad, Hercules, CA, USA). After the experiment, exosomes were localized in brain tissues by immunological staining for CD63 in the groups that had received exosome treatment.

2.2.6. Iron oxide for direct labelling of MSCs
MSCs were incubated for 30 min in 4 ml of RPMI media supplemented with 50 μm iron oxide. The cells were then centrifuged for 10 min at 2000 rpm to separate the iron-labelled MSCs. Labelled MSCs release exosomes that contain iron oxide. The labelled exosomes are created inside labelled MSCs and released. Direct labelling with iron oxide is simple and relatively physiological. In addition, genetic modification is not required [37].

2.2.7. Prussian blue staining
Cerebral sections were treated with acid solutions of ferrocyanides. Ferrocyanide reacts with ferric ions (+3) in tissues to produce ferric ferrocyanide, a bright blue pigment called Prussian blue. Iron-labelled injected MSCs were detected in cerebral tissue by Prussian blue staining using eosin as a counterstain [37].

2.3. Animals

Adult male albino rats (age=6–9 months; weight=250–270 g) were obtained from the Experimental Animal Unit of Cairo University's Faculty of Veterinary Medicine. Regular feed and flowing water were provided to the rodents in clean settings. The animals were subjected to laboratory conditions, including a pleasant room temperature (23 ± 3 °C) and bright illumination (12 h cycle starting at 8:00 AM). Animal testing required institutional review board permission (BUFVTM 23–03–22, Faculty of Veterinary Medicine, Benha University, Benha, Egypt). The National Institutes of Health (NIH) Publication 85–23, Revised 2011: Guide for the Care and Use of Laboratory Animals, was strictly followed during all investigations.

2.4. Induction of Alzheimer's disease (AD)

AD was induced by treatment with AlCl3 (17 mg/kg; once daily) dissolved in 5 ml of distilled water by intragastric intubation for 8 weeks [38]' [39].

2.5. Experimental design

After acclimating for one week, ninety adult rats were randomly divided into six groups. The five treated groups were considered statistically significant at a P value <0.05 comparing to the control group.

Group I (control; n= 35): The rats were divided equally into five subgroups.

Subgroup Ia: Standard laboratory conditions without any intervention.

Subgroup Ib: Intragastric intubation with 8 weeks of distilled water.

Subgroup Ic: Injected intraperitoneally with 5 % PEG-400/5 % Tween-80 (rapamycin vehicle) daily for 4 weeks.

Subgroup Id: Injected intraperitoneally daily with distilled water for 4 weeks.

Subgroup Ie: Single intraperitoneal injection of 0.2 ml of phosphate-buffered saline.

Group II (AD; n = 15): AD rats were sacrificed after 12 weeks of AD induction (at the end of the experiment).

Group III (AD + rapamycin; n= 10): After 8 weeks of AD induction, rats were injected daily with rapamycin intraperitoneally at a dose of 66 μl/10 g body weight for a final dose of 8 mg/kg [40].

Group IV (AD + MSC-exos; n=10): Eight weeks after AD induction, the rats were intraperitoneally injected with 0.5 ml of MSC-exos per rat at a concentration of 100 μg protein/ml [33].

Group V (AD + 3-methyladenine (3-MA) + chloroquine; n=10): After inducing Alzheimer's disease for 8 weeks, the rats were given 3-MA (10 mg/kg) and chloroquine (40 mg/kg) intraperitoneally once a day for 4 weeks [33].

Group VI (AD + MSC-exos + 3MA + chloroquine; n=10): Eight weeks after AD induction, the rats were treated with 3-MA (10 mg/kg) and chloroquine (40 mg/kg) daily until the end of the experiment. Simultaneously, a single dose of exosomes (0.5 ml, 100 μg protein/ml) was injected intraperitoneally [33].

2.6. Memory assessment using the novel object recognition test

A behavioural test known as the novel object recognition test (NOR) was used to investigate the neurological underpinnings of memory and learning in rats. It is founded on the inborn propensity of rats for novelty. Briefly, the rats are shown two identical things before having their memory tested again by switching out one of the familiar objects with a brand-new one. Recognition memory refers to the preference for exploring a novel object during a test rather than a familiar object [41].

During the final week of the experiment, the NOR test was used to evaluate memory. This was carried out in an open wooden box with a painted grey inside that measured 50 by 50 m and was 40 cm high. The replicas of the objects examined were constructed of glass, plastic, or metal and immobilized inside the arena [42].

The exam was divided into three phases: testing sessions, habituation, and familiarization.

Habituation: At the start of the last week of the experiment, the rats were permitted to independently explore the empty box for 5 minutes every day for 4 days.

Familiarization or sample phase: Twenty-four hours after the last habituation session, each rat was trained to recognize two identical objects in the test arena in two opposite and equally spaced locations for 7 minutes.

Proper testing session or choice phase: Twenty-four hours after the familiarization phase, the animals were again placed in the testing box for 7 minutes. A fresh object and one duplicate of each well-known item used during the sample phase were presented. After each session, the objects and the arena were washed with 70 % ethanol to prevent persistent olfactory stimulus preferences.

Performance measures and analyses: The act of pointing the nose at an object from a distance of two centimetres and/or touching it with the nose was referred to as object exploration. Conversely, turning around or sitting on the item was not regarded as an exploratory action.

The time the rat spent investigating objects during the sample phase and the choice phase was the

primary metric. To analyse the following metrics, a video camera was placed over the NOR arena, and behaviour was captured using a video tracking system.

e1: The total amount of time (in seconds) that the two identical objects were explored in the sample phase.

e2: The total amount of time (in seconds) spent per object in the decision phase (novel plus familiar).

d2: The discrimination ratio (novel familiar/new + familiar) is the difference in exploration time between novel and familiar objects (d1) divided by the total exploration time for the two objects in the decision phase (e2). This ratio accounts for any variations in the overall amount of exploration time.

RI: Recognition was measured using the recognition index (RI). The RI was calculated by dividing the time spent examining the well-known or novel object by the sum of the number of times both objects were explored, multiplied by 100.

2.7. Sampling

At the end of the trial period, the rats were given an intramuscular injection of ketamine (100 mg/kg) and xylazine (10 mg/kg) to induce anaesthesia after they had fasted for 12 hours. Separate groups of rats were used for histological examination and molecular analysis. After the rats were secured on the operating table, we fixed the left ventricular vasculature via perfusion. Histopathological methods, including electron microscopy, haematoxylin and eosin (H&E) staining, and immunohistochemistry (IHC) for glial fibrillary acidic protein (GFAP) and synaptophysin, were used to analyse the dissected brain tissue. Buffered formol saline (at 10 %) or 1 % glutaraldehyde (for electron microscopy) was used for fixation. Molecular analysis by real-time PCR and Western blotting was also performed on freshly removed brain tissue.

2.8. Gene expression profile

Following the manufacturer's instructions, TRIzol (Invitrogen) was used to extract total RNA from brain tissues. For miRNA isolation, a mirPremier microRNA Isolation Kit (Sigma Aldrich, USA) was used for extraction and purification. A NanoDrop 2000 C spectrophotometer was used to determine the concentration and purity of the extracted RNA (Thermo Scientific, USA). All samples had an A260/A280 absorbance ratio > 1.9, indicating high levels of RNA purity. Following the manufacturer's protocol, cDNA was synthesized from RNA using SensiFast cDNA synthesis kits (Sigma Bioline, UK). For miRNA, an NCode VILO miRNA cDNA Synthesis Kit (Invitrogen, USA) was used. Maxima SYBR Green/ROX qPCR master mix (2×) was used for quantitative PCR (Thermo Scientific, Catlog number: K0221 و USA) [43]. SYBR Green quantitative polymerase chain reaction (qPCR) was performed using the following conditions: 12.5 µl of Maxima SYBR Green qPCR Master Mix (Thermo Fisher Scientific), 0.3 mol/L of each forward and reverse primer, 500 ng of cDNA (except for the no template control and cDNA control), and 10 nmol/L of ROX Solution. A final volume of 25 µl was achieved by adding nuclease-free water to the mixture. All the primers used in this study were purchased from Genwez (New Jersey, USA) and are listed in Tables 1 and 2. Initial denaturation for 10 minutes at 95 °C was followed by 40 cycles of 15 seconds of denaturation at 95 °C followed by annealing/extension for 60 seconds at 60 °C in an AriaMx Real-Time PCR (Agilent Technologies, USA) to complete the reaction. After PCR, a melting curve analysis was carried out by cycling the temperature between 95 and 65 °C for 30 seconds. The expression levels of the target genes were normalized to those of GAPDH and U6, which served as housekeeping genes. Relative gene expression ratios (RQs) were calculated between the treated and control groups using the formula $RQ = 2^{-\Delta\Delta Ct}$ [44].

Table 1
Primer sequences for targeted genes.

Target gene	Nucleotide sequence (5′-3′)	Reference
iNOS	F: AGAGACGCTTCTGAGGTTCC	NM_012611.3
	R: CTGCACCAACTCTGCTGTTC	
NF-κB	F: GATCTCGACCTCCACCGGAT	NM_001008349.1
	R: TTCCCAGAGTTTCAGACGCC	
NeuroD1	F: CTGTCAGAGATCCTGCGCTC	NM_019218.3
	R: GCTGGGACAAACCTTTGCAG	
PS-1	F: CGTCCGTAGCCAGAATGACA	XM_039111826.1
	R: TACTCTGGGGTCGGCCATTA	
IDE	F: CACCTTGCACTCCATCCTCG	NM_013159.2
	R: TCTATTCTCTGGATGGCCGGA	
APP	F: ACCTCTCCAAGATGCAGCAG	XM_032900495.1
	R: CACCGATGGGTAGTGAAGCA	
mTOR	F: GCAATGGGCACGAGTTTGTT	NM_019906.2
	R: AGTGTGTTCACCAGGCCAAA	
AMPK	F: GAAGATCGGACACTACGTGCT	NM_023991.1
	R: ACTGCCACTTTATGGCCTGTC	
PTEN	F: CAATGTTCAGTGGCGGAACTT	NM_031606.2
	R: GGCAATGGCTGAGGGAACT	
P70S6K	F: GGGGCATTTACATCAAAAGGGG	NM_031985.1
	R: TGTGCGTGACTGTTCCATCA	
4EBP-1	F: ACTAGCCCTACCAGCGATGA	NM_053857.2
	R: AGCATCACTGCGTCCTATGG	
Beclin 1	F: GAATGGAGGGGTCTAAGGCG	NM_001034117.1
	R: CTTCCTCCTGGCTCTCTCCT	
LC3II	F: CCTGGGCCAGTCTTGTTAGG	NM_199500.2
	R: TGAGGGGCAAGATGGGTAGA	
P62	F: TTTGATTTGAGGCACCCCGT	NM_130405.2
	R: AGATTCAACCGCCATGTGCT	
BACE-1	F: AGACGCTCAACATCCTGGTG	NM_019204.2
	R: GGACAGCTGCCTTTGGTAGT	
GAPDH	F: AGTTCAACGGCACAGTCAA	XM_039107008.1
	R: TACTCAGCACCAGCATCACC	

Table 2
Primer sequences for targeted miRNAs.

Target miRNA	Nucleotide sequence (5′-3′)	Reference
miRNA-21	F: ACCAAAATGTCAGACAGCCCA R: CCACCTTGTCGGGTAGCTTA	NR_031823.1
miRNA-155	F: TTAATGCTAATCGTG R: CTCAACTGGTGTCGTGGAGTCGGCA ATTCAGTTGAGACCCCTAT	[45]
miRNA-126–3p	F: CAGCACATTATTACTTTTGGTACGC R: TGACCACGCATTATTACTCACG	NR_031871.1
miRNA-17–5p	F: ACACTCCAGCTGGGCAAAGTGCTTACAGTGC R: TGGTGTCGTGGAGTCG	
U6	F: CTCGCTTCGGCAGCACA R: AACGCTTCACGAATTTGCGT	XR_005491570.1

2.9. Western blot

For protein extraction from brain tissues, ice-cold 1X cell lysis buffer was used. Brain tissue was homogenized by being passed through a series of hypodermic needles (20 G, 22 G, and 26 G). After 30 minutes of agitation in ice water, the supernatant from the tissue lysates was collected by centrifugation at 12000 rpm. A spectrophotometer was used to determine the protein concentration using the Folin-Lowry method (Beckman Coulter Inc., Indianapolis, Indiana). Protein extracts were blended with Laemmli buffer and heated in a dry bath for 5 minutes at 95 °C. Protein samples (50 mg) were loaded onto 10 % sodium dodecyl sulfate (SDS) polyacrylamide gels for electrophoretic resolution. The purified protein was transferred to a polyvinylidene difluoride (PVDF) membrane for blotting (IPVH00010, Millipore, Merck, Germany). The blots were then incubated in 5 % nonfat dry milk in 0.1 % TBS/Tween 20 for 1 h at 4 °C to block further adsorption. After blocking, the membrane was incubated with the appropriate primary antibodies overnight at 4 °C. The membrane was then washed and incubated at room temperature for 1 hour with a secondary antibody conjugated to alkaline phosphatase (ALP). The protein bands were visualized by using a BCIP/NPT detection kit (BWR1067, Biospes, China). Densitometric analysis of the bands was conducted using ImageJ® software. Normalization was performed using anti-β-actin as a housekeeping protein.

2.10. Enzyme-linked immunosorbent assay for LC3II and P62

The brain expression of LC3II and P62 was measured using commercially available ELISA kits according to the manufacturer's instructions.

2.11. Histopathological analysis

2.11.1. H&E staining
Hippocampal tissues were removed from the brain and preserved in buffered formol saline (10 %). To prepare 4-μm-thick paraffin slices for haematoxylin and eosin (H&E) staining, Congo Red staining to show amyloid plaques and neuronal effects, and positively charged slides for immunohistochemistry (IHC), fixed tissue specimens were used. The fixed sections were dehydrated with increasing amounts of ethanol before being rinsed twice in distilled water and stained with H&E. Finally, using a light microscope (Leica DMR 3000; Leica Microsystems), two expert researchers who were blinded to the treatment groups examined, analysed, and imaged the histological sections [46].

2.11.2. Immunohistochemistry analysis
Paraffin sections were deparaffinized and hydrated. After blocking the endogenous activity of peroxidase using 10 % hydrogen peroxide, the sections were blocked for non-specific reactions and then incubated with primary (Anti-GFAP antibody (ab7260), Rabbit polyclonal to GFAP, 1/1000–1/2000 dilution, abcam, UK; Recombinant Anti-

synaptophysin antibody (ab14692), Rabbit polyclonal to synaptophysin, 1/100 dilution, abcam, UK; Anti-CD81 antibody (ab155760), Rabbit polyclonal to CD81, dilution 1/100–1/1000, abcam, UK). Then, after washing with phosphate buffer, biotinylated goat anti-rabbit secondary antibody was applied. For localization of immune-reaction, the slides were incubated with labelled avidin–biotin peroxidase, which binds to the biotin on the secondary anti-body. Diaminobenzedine was used as chromogen for visualization of the site of antibody binding which is convert-ed into a brown precipitate by peroxidase [47].

2.11.3. Morphometric study
Two experienced pathologists who were not aware of the experimental methods evaluated and rated the haematoxylin and eosin-stained brain sections. With at least two sections per animal, morphometric measurements were performed using ImageJ® software Version 1.52a28 and Fiji ImageJ software 48. Three distinct, nonoverlapping fields from each section were evaluated. The following criteria were evaluated: at x400 magnification, the dentate granule cell layer thickness and the total number of granule cells were measured; calibrations were performed with ImageJ's straight-line tool at the proper calibration (pixel-to-m ratio). Using ImageJ software version 1.52a28 and Fiji ImageJ software [48], morphometric analysis of the area fraction of Congo red-stained sections was performed. To isolate Congo red, the image type was modified to RGB Stack, which produced grayscale images of each channel. An image adjustment threshold was used to apply a threshold of 0–87 to the green channel. Then, using the analyse measure command, the area, area fraction, limit to the threshold, and display label were measured. The Allred score, which provides a scale of 0–8 (0–1 = negative, 2–3 = mild, 4–6 = moderate, and 7–8 = extremely positive), was utilized for IHC quantitative assessment. Using the QuPath program (0.1.2), the score was calculated by summing the proportion of positive cells and staining intensity grades (0–3) [49].

2.11.4. Transmission electron microscopy study
For vascular perfusion fixation through the left ventricle, glutaraldehyde (1 %) was utilized. Following dissection, rat brain tissues were incubated for two hours at room temperature in 0.1 M phosphate-buffered saline (PBS), pH 7.4, before being rinsed three times for ten minutes each with PBS. After the samples were postfixed for 30 min in 1 % osmic acid, they were cleaned three more times for 10 min each with PBS. For 30 minutes at each concentration, the fixed specimens were submerged in ethyl alcohol solutions (in ascending order) (30, 50, 70, 90 %, and absolute alcohol). Araldite 502 resin was used to preserve dehydrated specimens after they were soaked in acetone for an hour. The plastic moulds were divided into semithin pieces and then stained with 1 % toluidine blue using a Leica UCT ultramicrotome. Following semithin section analysis, ultrathin sections (50–60 nm thick) were created, uranyl acetate was used to stain them, and lead citrate was subsequently used as a counterstain. With the help of an electron microscope (JEOL-JEM-100 SX, Japan), the sections were further examined and photographed (10948426) at Tanta University.

2.12. Molecular interaction network and targeted pathways

All candidate genes identified in the present study were analysed following integrated transformation and correlation analysis using Funrich (version 3.3; http://www.funrich.org/) to identify molecular interaction networks and biological pathways under a threshold of P<0.05. To confirm the ameliorative effect of the target exosomal miRNAs, FunRich software was utilized for molecular pathway enrichment analysis for miRNA-21, miRNA-155, and miR-126–3p under a threshold of P<0.05.

2.13. Statistical analysis

To perform the statistical analysis, GraphPad Prism was used

reestablishment of important signalling pathways, such as those that control the formation and clearance of APP processing products, such as BACE1, PI3K/Akt, GSK-3β, AMPK, and IRS1. Numerous studies have suggested that BACE1 and rapamycin/mTOR interact closely [68], [69], [70]. GSK3β phosphorylation is promoted when mTOR is inhibited since it inactivates AKT, p70S6K, and p85S6K, three of its target kinases [71], [72].

Through mTOR suppression, AMPK signalling is a significant inducer of autophagy and is linked to a decrease in energy metabolism [65]. Decreased autophagic clearance, increased oxidative stress, and impaired metabolic control result from the loss of sensitivity of activated AMPK to cellular stress. The AMPK signal in the AD rat model decreased in accordance with this suggested scenario. Interestingly, the increase in AMPK expression in groups III and IV resulted from the inhibition of mTOR via the delivery of rapamycin and MSC-exos. Remarkably, earlier studies showed that AMPK activation might block mTOR signalling and induce autophagy by phosphorylating Ulk1 [66].

Additionally, we investigated how MSC-exos and rapamycin affected the clearance of tau. We discovered that both reduced the expression levels of PHF-1 and Tau, indicating a function for both phosphorylated and full-length Tau. Rapamycin administration did not result in decreased levels of hyperphosphorylated tau despite the reduction of tau kinases or enhanced autophagosome production, which is crucial for the removal of intracellular hazardous tau aggregates [73], [74]. Therefore, by modifying the expression of certain tau kinases and promoting the destruction of hyperphosphorylated tau via autophagy induction, the injection of rapamycin and MSC-exos decreased the degree of tau phosphorylation.

Taken together, these findings reveal that the administration of exosomes contributed to the preservation of the brain's PI3K/Akt and insulin IGF-1R signalling pathways, enabling defence against the age-related pathogenesis of AD.

We also assessed the role of sphingosine-1-phosphate (S1P) in controlling the effects of MSC-exos. Exosomes are defined as vesicles within multivesicular endosomes (MVEs) that are high in sphingomyelin and ceramide and are released when the MVE membrane merges with the plasmalemma [52]. Sphingolipids are implicated in brain ageing and neurodegenerative diseases such as AD, according to accumulating data [75], [76]. As dementia progresses, metabolic changes increase ceramide-dependent proapoptotic signalling and decrease neuro-protective S1P levels. Sphingolipids affect several facets of the Akt/-protein kinase B signalling system, which controls Bcl-2 family proteins, metabolism, and the stress response [8], [77]. By inhibiting neutral sphingomyelinase 2 (nSMase2), lowering the conversion of sphingo-myelin to ceramide, and eventually lowering the formation of amyloid plaques, exosomes contribute to the process of beta-amyloid peptide and tau clearance [78]. Exosome glycosphingolipids (GSLs) can bind to Aβ, hastening the removal of amyloid deposits and lowering synaptic toxicity [68]. According to Wang and Yang [69], a BM-MSC-Exo-based reduction in intraneural Aβ deposition markedly enhanced the spatial learning and memory capacity of APP/PS1 mice. Increased expression of S1K and S1P correlated with this improvement in cognitive performance, which was entirely reversed by intraperitoneal injection of S1K or S1P inhibitors. Group IV, which received MSC-exos treatment, exhibited noticeably increased S1P1 in the AD brain.

MSC-exos improved AD pathogenesis in ways in addition to modulating PI3K/AKT/mTOR signalling via S1P1. The inhibition of autophagy in AD mice by 3-methyladenine (3-MA) and chloroquine in Group V worsened AD pathogenesis. Group VI AD mice received MSC-exos with autophagy inhibitors, which moderately increased AD pathology. These findings suggested that MSC-exos may influence AD via different mechanisms.

Neuroinflammation is a key pathogenic process in AD caused by increased Aβ accumulation in the brain [70]. In pathological abnormalities, MSC-exos regulate immunity and reduce neuroinflammation [71]. The suppression of activated microglia, reactive astrocytes, and

cytokine release by MSC-exos causes anti-inflammatory effects [72]. MSC-exos also induce lymphocyte differentiation into an anti-inflammatory phenotype, decreasing T-cell differentiation into Th17 cells and increasing Treg numbers [45]. Nitric oxide synthase (NOS) in glial cells is induced by Aβ, leading to high levels of NO release. NO causes neurotoxicity by inhibiting mitochondrial respiration and killing neuronal cells [79]. In mouse models of AD, MSC-exos were shown to diminish Aβ-induced iNOS expression and improve cognitive function [80]. We found lower iNOS and NFK-ß gene expression in the rapamycin and exosome groups than in the AD group. GFAP immuno-reactivity in the rapamycin and MSC-exos groups was significantly lower than that in the AD group, confirming that inflammation induced astrogliosis.

According to Ma and colleagues, MSC-exos contain proteins that drive neurogenesis, myelin formation, neurite expansion and branching, axonal development and regeneration, and neuroprotection. RNA sequencing revealed 1094 upregulated and 267 downregulated genes in MSC-exos-treated neurons [81]. In experimental mice, MSC-exos boosted synaptic function gene expression and memory function. In AD mice, MSC-exos reduced neurologic damage, enhanced neuron generation, and restored memory [81]. Along with these findings, rapamycin and MSC-exos treatment increased NeuroD1 gene expression compared to that in the AD group, indicating that NSCs differentiated into neuronal cells. NeuroNeuroD1 is expressed by mitotic and early-postmitotic neuronal cells in the subventricular zone (SVZ) of the DG, which includes transit-amplifying progenitors that generate the most mature cortical excitatory neurons. Neuro D1 is expressed in the cerebral cortex postnatally. Synaptophysin immunoreactivity was greater in the rapamycin and MSC-exos groups than in the AD group, indicating improved synaptic function and cognition.

Recent findings further imply that exosomal small regulatory RNA pools modulate PI3K/AKT/mTOR signalling. CNS-abundant miRNAs can also regulate the expression of enzymes that produce ceramide, sphingosine, C1P, S1P, and/or their receptors in healthy brain ageing and neurological illness [52]. Tuberous sclerosis 1 (Tsc1), a negative regulator of mTOR kinase, is targeted by miR-126–3p, which affects cell survival and proliferation. The miR-17—92 cluster in MSC-exos activate the PI3K/Akt/mTOR signalling pathway, causing neuronal remodelling and neurogenesis in mouse models of cerebral stroke [75]. Proin-flammatory miRNA-155, produced by NF-κB, regulates S1PR1 production, alleviating pathogenic inflammation through Aβ clearance [76]. High miRNA-21 levels in MSC-exos reduce inflammation and apoptosis. In addition, exosomes from preconditioned MSCs effectively increased miR-21 levels and reduced NF-κB activation and STAT3 expression in AD animals. Overexpression of miR-21 improved memory and regulated pathology [8]. In additional disease models, MSC-exos with miR-142–3p, miR-223–3p, and miR-126–3p influence dendritic cell maturation and anti-inflammatory capability. By delivering miRNA-146a to macrophages, MSC-exos can decrease TRAF6 and IRAK1 expression, leading to reduced NF-κB phosphorylation and inflammatory factor expression. Some miRNAs in MSC-EVs have also been found to be therapeutic. Angiogenesis, cellular transport, proteolysis, and apoptosis involve miR-148a, miR-532–5p, and miR-378. Neural injury is associated with miR-21, miR-17–92, and miR-133b, while cellular differentiation is linked to miR-145 [77]. This study demonstrated that MSC-exos influence the PI3K/AKT/mTOR pathway via S1P signalling, boosting Aβ breakdown and modulating immunity, thereby improving neurological deficits. MSC-exos modulate microRNAs to facilitate AD progression. In this setting, MSC-exos may cure AD.

Conclusions

The present study provides evidence that MSC-exos modulate auto-phagy through the PI3K/AKT/mTOR pathway and related signalling pathways, promoting Aβ degradation, regulating immunity and leading to improved memory and neurological impairments. Furthermore, MSC-

exos regulate microRNAs to ameliorate AD pathogenesis. In this context, MSC-exos can be considered a potential therapeutic option for treating AD.

Funding

CRediT authorship contribution statement

Sabry Younis Mohamed Mahmoud: Visualization, Validation, Software, Formal analysis, Data curation. **Faris Q. Aleniz:** Visualization, Validation, Software, Resources. **Ola Mostafa:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Arigue A. Dessouky:** Software, Resources, Methodology, Formal analysis, Data curation. **Ayman Samir Farid:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Noha I. Hussien:** Writing – original draft, Resources, Methodology, Formal analysis, Data curation. **Omnia A. Badr:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Rabab F. Salim:** Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Mohamed El-Sherbiny:** Writing – review & editing, Writing – original draft, Visualization, Validation, Resources. **Hajer A. Al Saihati:** Visualization, Supervision, Software, Resources, Formal analysis. **Fares E.M Ali:** Software, Methodology, Investigation, Formal analysis, Data curation. **Nesrine Ebrahim:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Nicholas Robert Forsyth:** Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources. **Zahraa Alali:** Writing – original draft, Visualization, Validation, Software, Resources. **Nimer F. Alsabeelah:** Writing – review & editing, Validation, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

Institutional review board statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH publication No. 85–23, revised 1996). The institutional animals' care approved all protocols and used the committee and research ethics board of the Faculty of Veterinary, Benha University, Egypt. The project title is IACUCREB, the approval number is BUFVTM, and the approval date is 23/3/2022.

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