Protective Effect of Exosomes Derived from Bone Marrow Mesenchymal Stem Cells Against AD-Like Behaviors in Mice: Involvement with Regulating Glial Activation and its Associated Neuroinflammation and BDNF-Related Neuropathological Changes in the Hippocampus

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Research

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Abstract

**Background:** Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive decline in cognitive ability. Exosomes derived from bone marrow mesenchymal stem cells (BMSC-exos) are extracellular vesicles that can execute the function of bone marrow mesenchymal stem cells (BMSCs). Given the versatile therapeutic potential of BMSC and BMSC-exos, especially the neuroprotective effect, the aim of this study is to investigate the potential effect of BMSC-exos on the AD-like behavioral dysfunction in mice and explore the possible molecular mechanism.

**Methods:** BMSCs were isolated from the femur and tibia of adult C57BL/6 mice, purified and sorted via flow cytometry technology, and cultured *in vitro*. The BMSC-exos were extracted and identified via transmission electron microscopy, and Western Blot was used to detect exosomes labeled proteins. A sporadic AD mouse model was established by intracerebroventricular injection of streptozotocin (STZ). 6 weeks later, BMSC-exos were given via lateral ventricle injection or caudal vein injection lasting for five consecutive days, and the control mice were intracerebroventricular given equal volume of solvent. The behavioral performance were observed via the Open field test (OFT), elevated plus maze test (EPM), novel object recognition test (NOR), Y maze test (Y-maze), and tail suspension test (TST). The mRNA and protein expressions of IL-1β, IL-6, and TNF-α in the hippocampus were measured via quantitative polymerase chain reaction (QPCR) and Western Blot, respectively. Meanwhile, the protein expression of Aβ1-42, BACE, p-Tau (Ser396), Synaptotagmin-1, Synapsin-1, and brain-derived neurotrophic factor (BDNF) in the hippocampus was detected using Western blot, and the expression of GFAP, IBA1, and DCX in the hippocampus was measured via immunofluorescence staining.

**Results:** Lateral ventricle administration, but not caudal vein injection of BMSC-exos could improve the AD-like behaviors in STZ-injected mice model, as indicated by the increased number of rearing, increased frequency to the central area, and increased duration and moving distance in the central area in the OFT, together with improved preference index of the novel object in the NOR. Moreover, the hyper-activation of microglia and astrocytes in the hippocampus of the model mice were inhibited after treatment with BMSC-exos via lateral ventricle administration, accompanied by the reduced expression of IL-1β, IL-6, TNF-α, Aβ1-42, and p-Tau, and upregulated protein expression of synapse-related proteins and BDNF. Furthermore, results of Pearson test showed that the preference index of the novel object in the NOR was positively correlated to the hippocampal expression of BDNF, but negatively correlated with the expression of GFAP, IBA1, and IL-1β. Apart from a positive correlation between the hippocampal expression of BDNF and Syt-1, BDNF abundance was found negatively correlated with the markers of glial activation and the expression of inflammatory cytokines, Aβ, and p-tau, which were characteristic neuropathological features of AD.

**Conclusions:** Lateral ventricle administration, but not caudal vein injection of BMSC-exos can improve the AD-like behavioral performance in STZ-injected mice, the mechanism of which might be involved with regulating glial activation and its associated neuroinflammation and BDNF-related neuropathological changes in the hippocampus.
Background

Alzheimer's disease (AD) is a neurodegenerative disease characterized by progressive cognitive decline, accounting for about 50–60% of all dementias [1]. Although the investigations targeting at the pathogenesis and treatment of AD has been lasting for more than a century, the etiology of AD is still controversial [2]. The main and more seasoned hypotheses are the amyloid and tau hypotheses that have undergone many debates over the last some decades. It has been demonstrated that the overproduction of amyloid β peptide could result in the hyper-phosphorylation of Tau, together with which it may lead to the altered synaptic function, increased inflammatory responses and oxidative injury, and altered kinase and phosphatase activities, eventually resulted in the deposition of amyloid plaques and neurofibrillary tangle formation. However, focusing on amyloid and neurofibrillary tangle formation, a considerable number of clinical trials of anti-AD drugs have mostly ended in failure [3]. More recently, the U.S. Food and Drug Administration (FDA) granted accelerated approval for Aducanumab, which is the first new drug for AD targeting at amyloid β. However, its effect remains controversial, and a new randomized controlled clinical trial was required to conduct by the FDA to examine the clinical benefits of the drug [4]. Thus, there is room to bring in novel hypotheses that represent the entire scope of the pathological features of AD, and it is imperative to explore the potential therapeutic methods.

Inflammation is one of the indispensable factors for cognitive impairment, contributed to the deposition of amyloid plaques, or cause neural death or synaptic dysfunction alone [5–8]. The study of Nikolaos et al. showed that the level of Interleukin 1β (IL-1β) in the monocyte culture supernatant of AD patients was significantly increased [9]. Excessive IL-1β can promote the synthesis and processing of APP and the activation of astrocytes, and further aggravate the degree of inflammation, forming a vicious circle. Interleukin 6 (IL-6) is another inflammatory factor with multiple functional effects. It plays an important role in the growth and differentiation of central nervous system cells, and inflammation and immune response. Harald et al. found that the level of IL-6 mRNA in the hippocampus of AD patients was significantly increased, and IL-6 also participated in and promoted the synthesis of APP [10]. In the early stages of AD, Aβ can be phagocytosed and cleared by microglia, but with the pathological development of AD, this phagocytosis will be weakened, which in turn leads to an increase in Aβ accumulation [11]. Aβ that is far above the normal level will stimulate the activation of astrocytes and microglia to produce inflammatory factors and toxic molecules. In addition, IL-1β secreted by microglia after activation will promote the expression of acetylcholin esterase (ACHE), which will lead to the decline of cholinergic function and aggravate the progress of AD [12]. These results indicated that neuroinflammation, alone or in conjunction with the increased Aβ and Tau, play an important role in the development process of AD [13–15].

Synapse loss is considered to be a downstream effect of Aβ and Tau and other AD pathogenesis [16]. In AD mice and cell models, Aβ is reported to be combined with synapses to destroy the normal activities of synapses, and induce abnormal synapses in morphology and number [17, 18]. It has also been demonstrated that the expression levels of brain-derived neurotrophic factor (BDNF) and synapse-related protein synaptotagmin-1 and Synapsin-1 in the hippocampus of AD model rats are significantly reduced.
[19], and the simultaneous decrease of synapse-related protein and BDNF levels may aggravate the process of AD.

Exosome is a type of extracellular vesicles with a diameter of about 30 ~ 200nm, which carry important genetic information such as mRNA, microRNA (miRNA) and proteins. The information transmission and communications between cells can be mediated by exosomes [20]. Compared with that in the normal population, the abundance and the miRNAs profiles of the exosomes was significantly changed in the plasma and cerebrospinal fluid of patients with neuropsychiatric diseases, including AD and depression [21–23]. In line with these findings, results of our previous studies have demonstrated a different miRNA expression profile of serum exosomes in depression-like and the control ones [24], suggesting that exosomes and their contents play different roles under different physiological conditions.

Bone marrow mesenchymal stem cells (BMSCs) are typical stem cells that can be differentiated into different cells under different physiological conditions, and they can selectively migrate to the site of tissue damage, and interact with brain cells, and then stimulate the production of nutritional factors such as BDNF and nerve growth factor (NGF) [25–27]. BMSCs are considered to be the most effective exosomes producing cells [28]. As compared with BMSCs themselves, exosomes derived from BMSCs (BMSC-exos) have more advantages that they will not replicate or cause proliferation so there is no risk of inducing tumor formation, and they will not undergo metabolism, not affect the use environment, and not be affected by the environment as well. Most importantly, BMSC-exos have similar effects to BMSCs without side effects [29–31]. Targeting at the biological functions of BMSCs and BMSC-exos, there are many in-depth and extensive research, with the results of beneficial effects against many diseases including heart diseases [32, 33], kidney injuries [34], ulcer [35], and so on. For example, miR-486 carried by exosomes derived from adipose-derived stem cells can be used as an autophagy activator to transfer into podocytes, and reduce the cell damage of MPC5 cells in diabetic mice [34]. The study of Dittmer et al. found great advantages of mesenchymal stem cells in promoting ulcer wound healing, while exosomes released by mesenchymal stem cells and microvesicles were reported to play a key role in wound healing [35]. More recently, the therapeutic effect of mesenchymal stem cell exosomes against neuropsychiatric diseases has also been demonstrated. It has been reported that the neuroinflammation and neuronal apoptosis in primary neurons of transgenic AD mice could be reduced the treatment of exosomes derived from mesenchymal stem cells [36]. Moreover, Long et al. found that exosomes derived from mesenchymal stem cells were administered intranasally could reduce inflammation and prevent abnormal neurogenesis and memory dysfunction after status epilepticus, but its mechanism of action needs to be further elucidated [37]. However, the effect of BMSC-exos on neuroinflammation and cognitive decline in AD animal models has not yet been elucidated.

To investigate the potential effect of BMSC-exos against AD-like behavior, a sporadic-like AD mouse model was established in the present study by intracerebroventricular injection of STZ, and BMSC-exos were given six weeks later via lateral ventricle injection or caudal vein injection. The behavioral performance was observed by OFT, EPM, Y-maze, NOR, and TST. Nissl staining was used to confirm the injection site of exosomes. Glial cell activation and new neurons in the hippocampus were observed by IF.
The protein expression levels of IBA1, GFAP, IL-1β, IL-6, TNF-α, BACE, Aβ1-42, p-Tau, Tau5, Synaptotagmin-1, Synapsin-1, and BDNF in hippocampus were detected via Western blot, and the mRNA expression levels of IL-1β, IL-6 and TNF-α were measured by QPCR technique.

Materials And Methods

Cell culture medium

BMSCs complete medium consists of α-MEM basic medium (BL306A, biosharp) plus 10% fetal bovine serum (04-001-1ACS, Biological Industries), 1% penicillin-streptomycin solution (C0222, Beyotime), 20ng/mL EGF (50482-MNCH LC13JA1403, Sino Biological), and 20ng/mL bFGF (50177-M08H LC12SE1702, Sino Biological).

Isolation, culture and purification of primary BMSCs

The adult male C57BL/6 mice were sacrificed by cervical dislocation and then sterilized and immersed in 75% ethanol for 5-10 minutes. The femur and tibia were separated under aseptic conditions and cut into pieces with a volume less than 3mm³. Red blood cells were lysed with red blood cell lysate (C3702-120mL, Beyotime), and after centrifugation, the fragments were re-suspended in culture medium and inoculated in a culture flask, and placed in an incubator at 37°C, 5% CO₂ and 95% O₂ with saturated humidity. Cells were passaged each time when the cells form a certain number of colonies in the culture flask. The third-generation cells which were incubated with APC-CD44 (103012, Biolegend), FITC-CD45 (103107, Biolegend) and PE-CD11b (101207, Biolegend) were used for flow sorting, and the cells of CD44⁺/CD45⁻/CD11b⁻ were sorted by Beckman flow cytometry sorting system (Moflo-XDP, Backman). The sorted cells were collected in a sterile flow tube, transferred to a cell culture flask to continue culturing, and replaced the culture medium every 48h.

Extraction, separation, and fluorescent labeling of BMSC-exos

After the sorted cell culture reaches a certain density, the complete culture medium was replaced by an exosomal-free serum, and the cell supernatant was collected every 48h. The exosomes were extracted using the cell supernatant exosome isolation kit (4478359, Thermo Fisher Scientific), resuspended with PBS, and freezed at -20°C for later use. The BCA protein quantification method was used to quantify the extracted exosomes, the expression of exosomal marker proteins was detected by western blot, and the PKH26 staining kit (MX4021-100UL, Maokangbio) was used to stain and label the exosomes. In preparation for injection via lateral ventricle or caudal vein, the exosomes were resuspended and collected with an appropriate amount of pre-chilled artificial cerebrospinal fluid (ACSF).

Animals

Twenty four male C57BL/6 mice aging 4-weeks were purchased from the Anhui Animal Center. The breeding conditions were 12h alternating light and dark, free to food and water, with ambient temperature
20±2°C and humidity 50±5%. When the mice grew to 12 weeks of age, they were randomly divided into a control group (6 animals) and an STZ group (18 animals), with three animals in each cage. All animal manipulation procedures have been approved by the Experimental Animal Ethics Committee of Anhui Medical University and comply with the "Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health" (NIH Publication No. 85-23, revised in 1985).

**Establishment of animal model and experimental design**

All mice were anesthetized by intraperitoneal injection of 5% chloral hydrate (0.1mL/10g), their heads were skin-prepared and disinfected, and then fixed on a stereotaxic apparatus. A 0.8-1cm incision was made in the center of the scalp. Suitable 3% H$_2$O$_2$ was used to digest and erase the mucosa on the surface of the skull until the Bregma was exposed, then the surface of the skull was wiped with normal saline, so that the surface of the skull was clearly exposed. The surface of the mouse skull was adjusted to a level, with fontanelle as the origin of the coordinates, and located the bilateral ventricles (the coordinates are 1.0mm left and right of the Bregma, 0.5mm back, and 2.5mm deep). The electric cranial drill was used to drill the hole, then the STZ was injected into the lateral ventricle of mice by the autosampler in the STZ group (the injection dose was 0.3mg/kg, the injection speed was 0.5μL/min, the injection volume was 1μL/side, and the STZ was dissolved in ACSF, and it was prepared for current use). Mice in the control group were given ACSF with an equal volume. At the end of the injection, the needle was stopped for 5 minutes and then the needle was withdrawn, the mouse scalp was sutured, and an appropriate amount of normal saline was injected into the intraperitoneal cavity, and then transferred to an electric blanket until awakened and placed in a squirrel cage.

After a one month recovery period for all the mice, the STZ-injected mice were divided into 3 groups including a model group, an exosomes lateral ventricle injection (Lv) group, and an exosomal caudal vein injection (Cv) group, with 6 mice in each group. The right cerebral ventricle of all mice was intubated using a stereotaxic instrument and a drug delivery catheter (62003, 62102, RWD). The mice in the Lv group were injected with 0.5μg BMSC-exos (dissolved in ACSF) per day in the lateral ventricle, the control group and the model group were injected with equal volumes of ACSF, and the mice in the Cv group were injected with 25μg BMSC-exos (dissolved in PBS) per day in the tail vein. All mice were administered for 5 days. After the administration, a series of behavioral tests were performed on all mice. The experimental time and steps are shown in Figure. 1.

**Behavioral tests**

All behavioral tests were performed under dark, sound-proof conditions. All mice were given 30 minutes to adapt to the environment before the behavioral experiments. All of the tests were carried out between 08:30 and 12:30, with matching between the groups. The observers were blind to the treatment. The behavioral tests were monitored and recorded by a digital camera interfaced to a computer running the ANY-maze video imaging software (Stoelting Co, Wood Dale, American).

**Open field test (OFT)**
The experimental apparatus of the OFT is a cube white opaque box with a size of 45cm×45cm×45cm. Each mouse was put into the box with its back facing one side of the box wall and allowed to explore freely for 5 min.

**Elevated plus maze test (EPM)**

The EPM apparatus consists of two opposite open arms (50×10×0.5cm), two opposite closed arms (50×10×20cm), and a central open platform (10×10cm). Each mouse was placed on the central platform facing one of the open arms, and allowed to explore the instrument freely for 5 minutes. The moving distance of each mouse in the closed arm is counted.

**Y maze test (Y-maze)**

The Y-maze apparatus is composed of three white opaque plexiglass arms. The size of each arm is 40cm×20cm×10cm. This task consisted of 2 sessions (training and test) and conducted on 2 successive days. In the training session, the novel arm was closed, and the mice were placed at the end of the starting arm facing the wall and allowed to explore in the maze for 10 min. 24 h later, the test session was performed. The novel arm was opened, and the mice were permitted to explore within the three arms for 5 min. The ambulatory distance in each arm was recorded and the ratio of moving distance in the novel arm to that in the total arm was taken as the preference index of the novel arm.

**Novel object recognition test (NOR)**

The NOR apparatus is a cube white opaque box with a size of 45cm×45cm×45cm. The experiment is divided into two stages. The first three days are the first part (adaptation period). Each mouse was put into the apparatus with facing one side wall every day, explored and adapted freely for 10 minutes. The fourth day is the second part (the inspection period). The inspection period is divided into two stages. The first stage: two objects of the same shape, size, and color were placed on the two thirds of the diagonal of the bottom of the box. Each mouse was put into the instrument facing one side, explored freely for 10 minutes, and entered the second stage after 1 hour. The second stage: one of the objects was replaced by a novel object with different shapes and colors, each mouse was put back into the apparatus and explored freely for 5 minutes. The behavioral software was used to record the mouse's behavior on the novel and old objects within 5 minutes. Calculating the mouse's preference for novel and old objects, that is, the novel object preference index: time to explore novel objects/total time to explore novel and old objects, and perform statistical analysis.

**Tail suspension test (TST)**

The TST apparatus is a 40 cm high white open box. The 1.5cm part of the tail end of the mouse is fixed with paper tape and hung on a crossbar directly above the box. The experiment process was recorded by an automatic camera. Each mouse was hung for 6 minutes. The first two minutes is the adaptation period, and the last 4 minutes is the official tail suspension time. The immobility time of the mice in the last 4 minutes was observed and recorded through video observation. The judgement was based on
keeping the two front paws of the mouse as the standard, and all the results were recorded by the same person.

**Transmission electron microscope observation of BMSC-exos morphology**

A small amount of exosomal suspension was put on the parafilm, and the exosomes in the suspension was absorbed with the sample-loading copper mesh for 3 minutes, then it was stained with 2% phosphotungstic acid negative staining solution for 3 minutes. At last, the sample was transferred to a transmission electron microscope for observation and image collection.

**Immunofluorescence staining (IF)**

Three mice in each group were randomly selected for cardiac perfusion, perfused with PBS until there was no more blood outflow, replaced with 4% paraformaldehyde perfusion, and reperfused for 3 minutes after the systemic spasm. The whole brain of each mouse was separated completely and fixed overnight in 4% paraformaldehyde solution at room temperature, then immersed in 30% sucrose solution and dehydrated to the bottom. The whole brain was filled with a disposable embedding box, filled with OCT embedding solution and frozen at -80°C overnight. Slice with 30μm-thick were prepared with a cryostat, attached to a glass slide, then returned to room temperature and dried in a fume hood for 10 minutes. The tissue was surrounded with an immunohistochemistry pen, and the goat serum (ZLI-9022, ZSGB-BIO) was used to seal the sections at room temperature for 30 minutes. After the liquid was cleaned, the sections were incubated with the diluted rabbit anti-GFAP (1:100, 16825-1-AP, Proteintech), rabbit anti-DCX (1:100, 13925-1-AP, Proteintech), or rabbit anti-Iba-1 (1:100, DF6442, Anity) overnight at 4°C in the dark. After washing with PBST, the sections were incubated with the goat anti-rabbit FITC or goat anti-rabbit Cy3 at 37°C for 1 hour. After washing with PBST, the tablets were sealed with anti-Fluorescence quenching mounting tablets containing DAPI (S2110, Solarbio), then the plates were mounted with a laser scanning confocal fluorescence microscope (LSM800, ZEISS) for imaging.

**Nissl stain**

The slices of the ventricles were degreased with xylene, stained with Nissan staining solution to dark blue, washed with double distilled water, and an appropriate amount of differentiation solution was added dropwise for differentiation. Then dehydrated with gradient alcohol, degreased again with xylene, and finally sealed with neutral gum. The slices were placed in a fume hood to dry for 2 hours, then observed and collected images with a microscope.

**Western blot assays**

**Exosomal marker protein detection**

The exosomal suspension was mixed with SDS protein loading buffer for Western blot experiments to detect the surface marker protein anti-CD63 (1:1000; sc-5275, Santa Cruz), anti-TSG101 (1:1000;
Detection of protein expression in mouse hippocampus

In each group, 3 mice were selected immediately after cervical dislocation and sacrificed. The brains were dissected immediately, and hippocampal tissues were isolated and stored in liquid nitrogen. The Western blot method is consistent with our previous research, using RIPA (P0013B, Beyotime) lysate containing protease inhibitors and phosphatase inhibitors to lyse the tissues, and using the BCA protein quantification kit (P0010, Beyotime) to determine the protein concentration in each sample. The protein loading buffer and protein sample were mixed in boiling water and heated for 10 minutes. After cooling, the sample was added to 12.5% SDS-PAGE. After electrophoresis, the membrane was transferred and the protein was quickly blocked. The primary antibody was incubated overnight at 4°C. The main antibody dilution ratios are as follows: anti-Aβ1−42 (1:1,000; 25524-1-AP, Proteintech), anti-Tau5 (1:1000; sc-58860, Santa Cruz), anti-p-Tau (ser396) (1:1,000; sc-32275, Santa Cruz), anti-BACE (1:500; WL02795, Wanleibio), anti-GFAP (1:1000; 16825-1-AP, Proteintech), anti-Synaptotagmin-1 (1:1000; YT4484, Immuno Way), anti-Synapsin-1 (1:1000; BS3667, Bioworld), anti-BDNF (1:1000; ab108319, Abcam), anti-IL-1β (1:500; WL00891, Wanleibio), anti-IL-6 (1:1000; WL02841, Wanleibio), anti-TNF-α (1:1000; WL01581, Wanleibio), and anti-β-actin (1:1000; TA-09, ZSGB-BIO). The membranes were then processed with appropriate HRP-conjugated secondary antibodies with regard to the proteins of interest. The protein levels were analysed using ImageJ (Wayne Rasband, National Institutes of Health, USA) and normalized relative to that of the internal control β-actin.

Quantitative Real-time PCR (QPCR)

Quantitative real-time polymerase chain reaction (QPCR) was performed to determine IL-1β mRNA, IL-6 mRNA and TNF-α mRNA levels in all experimental groups. Part of the hippocampus from three mice in each group was used to extract RNA. The extraction steps are as follows. In short, the Trizol (15596026, ambion) was used to lyse the tissues and add chloroform to incubate and then centrifuge, then the supernatant was mixed well with isopropanol, and precipitated overnight at -20°C. The supernatant was discarded after centrifugation and the precipitate was resuspended with 75% ethanol. The supernatant was discarded after centrifugation, and the precipitate was resuspended in enzyme-free water, and performed RNA quantification. After quantification is completed, the Evo M-MLV RT Premix (AG11706, Accurate Biology) was used to reverse transcription, and then use cDNA, upstream primers, downstream primers, enzyme-free water and SYBR Green (AG11701, Accurate Biology) for real-time fluorescence quantification. PCR was performed in a thermal cycler as follows: 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s, and 55 °C for 30 s. IL-1β-F primer sequence was 5'CTTTGAAGTTGACGGACCC3', IL-1β-R primer sequence was 5'TGAGTGATCTGCTGCCTG3', IL-6-F primer sequence was 5'AGTCCGAGAGGAGACTTCA3', IL-6-R primer sequence was 5'ATTTCACGATTTCCAGAG3', TNF-α-F primer sequence was 5'CACCACCATCAAGGACTTCA3', TNF-α-R primer sequence was 5'AGGCAACCTGACCACCTC3', β-actin-F primer sequence was 5'AGTGTGACGTTGACATCGT3', β-actin-
R primer sequence was 5'TGCTAGGAGCCAGAGCAGTA'. The experimental results are calculated using the $2^{\Delta\Delta CT}$ method and normalized analysis.

**Statistical Analysis**

All statistical analyses were performed using SPSS (Statistical Package for the Social Sciences, version 17.0). Data are expressed as the mean ± S.E.M., and p < 0.05 was considered statistically significant. Intergroup statistical analysis was performed by one-way analysis of variance (ANOVA) followed by the LSD post hoc test. All statistical charts are drawn using Graphpad Prism 7.0 and Microsoft Powerpoint 16.0.

**Results**

**BMSCs sorting and cultivation**

As shown in Figure 2A, after sorting and purification, BMSCs proliferated normally and grew fibrous and colony-like.

**Identification And Fluorescent Labeling Of Bmsc-exos**

The CD44-positive (CD44⁺, 61.0%), CD45 and CD11b-negative (CD45⁻, CD11b⁻, 96.9%) cells were marked in the flow cytometry, and CD44⁺/CD45⁻/CD11b⁻ BMSCs were sorted by flow cytometry sorting system (Figure 2B). BMSC-exos were extracted using a commercial kit. Under transmission electron microscope, the BMSC-exos showed a typical double-layer membrane and cup holder-like structure with a particle size of about 50nm (Figure 2C). The exosomal marker proteins CD63, HSP70, and TSG101 were highly expressed (Figure 2D). After stained with PKH26 reagent, a red circle could be found under a confocal microscope (Figure 2E).

**Exosomes are successfully injected into the ventricles and can be delivered to the hippocampus**

The results of Nissl staining showed that the delivery catheter was accurately positioned to the lateral ventricle, confirming the delivery location of BMSC-exos, which was shown in Figure 3A. The results of IF showed that certain PKH26-labeled red exosomes could be detected in the hippocampus of Lv group but not in other groups, confirming that BMSC-exos can be delivered to the hippocampus, as shown in Figure 3B and 3C.

**Lateral ventricle administration, but not caudal vein injection of BMSC-exos, improved the AD-like behaviors in STZ-injected mice model**

Figure 4 shows the behavioral performance of all the mice in this study. Although there was no statistical difference among groups as regard to the total ambulatory distance in the OFT (Fig. 4A), the STZ intracerebroventricularly injected model mice showed less number of line crossed (Fig. 4B), less frequency (Fig. 4C), less moving distance (Fig. 4D), and less number of rearing (Fig. 4E) in the central area than the control ones. These changes were reversed in Lv group, but not Cv group (Fig. 4B-D). In the
EPM test, there was no statistical difference among groups of the moving distance in the closed arm (Fig. 4F). Compared with that of the control mice, the preference index of the novel object in the NOR test (Fig. 4G) and the preference index of the novel arm in the Y maze (Fig. 4H) of the model mice were both decreased, while the immobility time in the TST (Fig. 4I) was increased. Lateral ventricle administration of BMSC-exos could increase the preference index of the novel object in the NOR test (Fig. 4G), but has no significant effect on the preference index of the novel arm in the Y maze (Fig. 4H) and the immobility time in the TST (Fig. 4I). Typical trajectories of mice in the OFT, EPM, NOR, and Y-maze are shown in Fig. 4J.

Results of the Pearson's correlation test showed that the preference of novel object in the NOR was positively correlated to the duration in the central area in the OFT ($r = 0.7486, P = 0.0051$, Fig. 4K), distance in the center in the OFT ($r = 0.7565, P = 0.0044$, Fig. 4L) and the number of rearing in the OFT ($r = 0.6487, P = 0.0225$, Fig. 4M).

**Lateral ventricle administration, but not caudal vein injection of BMSC-exos, inhibited the microglia activation and neuroinflammation in the hippocampus of STZ-injected AD mice model**

Figure 5 and Fig. 6 shows the typical graphs and the statistical analyses of the expression of IBA1 and GFAP in the hippocampus of mice. Compared with that of the control mice, the number of IBA1 and GFAP positive cells and the protein expression of IBA1 and GFAP in the hippocampus of the model group were increased significantly, which were reversed in the Lv group, but not the Cv group. Unsurprisingly, results of Pearson test showed that the hippocampal expression of GFAP was positively correlated to expression of IBA1 ($r = 0.9311, P < 0.0001$).

As shown in Fig. 7, the protein (Fig. 7A, 7B) and mRNA (Fig. 7C) expression levels of inflammatory factors IL-1β, IL-6 and TNF-α in the hippocampus of the model mice were all remarkably increased as compared with the control ones, which were reversed by treatment with BMSC-exos via lateral ventricle administration, but not caudal vein injection.

**Lateral ventricle administration, but not caudal vein injection of BMSC-exos, reduced the amyloid accumulation and Tau hyperphosphorylation in hippocampus of STZ-injected AD mice model**

Compared with that of the control mice, the expression levels of BACE, Aβ$_1$-42 and p-Tau (ser396) in the hippocampus of the model group were significantly increased (Fig. 7D, 7E). There was no significant difference between the model and the Cv group, but the expression of these proteins was decreased significantly in the Lv group (Fig. 7D, 7E).

Results of the Pearson's correlation test showed that the hippocampal expression of IL-1β was positively correlated not only to the hippocampus expression of IL-6 and TNFα ($r = 0.8646, P = 0.0003$, Fig. 8B; $r = 0.9545, P < 0.0001$, Fig. 8C), but also to the expression of IBA1 ($r = 0.8236, P = 0.0010$, Fig. 8A). A positive correlation was also found between the hippocampal expression of Aβ$_1$-42 and BACE or p-Tau ($r = 0.8437, P = 0.0006$, Fig. 8G; $r = 0.7711, P = 0.0033$, Fig. 8H). Additionally, the preference index of novel object in the NOR was negatively correlated to the expression of GFAP, IBA1, IL-1β and Aβ$_1$-42 ($r = -0.7344, P = 0.0065$, ...
Fig. 8D; r=-0.7037, P=0.0107, Fig. 8E; r=-0.889, P=0.0001, Fig. 8F; r=-0.8041, P=0.0016, Fig. 8I) in the hippocampus.

**Lateral ventricle administration, but not caudal vein injection of BMSC-exos, promoted neuron regeneration and the expression of BDNF in the hippocampus of STZ-injected AD mice model**

Figure 9A and 9B show the number of DCX positive cells in the hippocampus of each group. Compared with the control group, the model mice presented reduced number of DCX positive cells in the hippocampus. The number of DCX positive cells in the Lv group was significantly higher than that of the model group, with no significant difference between the model group and the Cv group.

As shown in Figure 9C and 9D, the expression levels of Synaptotagmin-1, Synapsin-1, and BDNF in the model group were significantly decreased as compared with that of the control ones. Compared with the model group, the expression level of BDNF increased significantly in the Lv group, but not the Cv group. The expression levels of Synaptotagmin-1 and Synapsin-1 were decreased in the model group, but not changed significantly after treatment with BMSC-exos, no matter by means of lateral ventricle administration or caudal vein injection.

Results of the Pearson's correlation test showed that the hippocampus expression of BDNF was positively correlated with the number of rearing in the OFT, the preference of novel object in the NOR, and the hippocampus expression of Syt-1 to (r = 0.6801, P = 0.0150, Fig. 10A; r = 0.81, P = 0.0014, Fig. 10B; r = 0.7865, P = 0.0024, Fig. 10C), but negatively correlated with the protein expression of IBA1, IL-1β, and Aβ1-42 in the hippocampus(r=-0.8168, P = 0.0012, Fig. 10D; r=-0.8646, P = 0.0003, Fig. 10E; r=-0.8472, P = 0.0005, Fig. 10F).

**Discussion**

In the present study, we investigated the potential effect of BMSC-exos against AD-like behaviors in a STZ-injected mice model, and the results showed that lateral ventricle administration of BMSC-exos could improve the AD-like behaviors in STZ-injected mice model, as indicated by the increased motion and rearing in the OFT, together with an increased preference index of novel object in the NOR. Moreover, hippocampal inflammation and hyper-activation of glial cells in the AD mice model was significantly improved after lateral ventricle administration of BMSC-exos, and the protein expression level of BACE, Aβ1-42 and p-Tau were significantly reduced, while the expression level of BDNF was significantly increased. Furthermore, results of Pearson test showed that the preference index of the novel object in the NOR was positively correlated to the hippocampal expression of BDNF, but negatively correlated with the expression of GFAP, IBA1, and IL-1β. Apart from a positive correlation between the hippocampal expression of BDNF and Syt-1, BDNF abundance was found negatively correlated with the markers of glial activation and the expression of inflammatory cytokines, Aβ, and p-tau, which were characteristic neuropathological features of AD. These results indicate that lateral ventricle administration, but not caudal vein injection of BMSC-exos, can improve the AD-like behaviors in STZ-injected mice, the
mechanism of which might be involved with regulating glial activation and its associated neuroinflammation and BDNF-related neuropathological changes in the hippocampus.

According to the latest statistics from the Alzheimer’s Association of the United States, as of 2020, more than 50 million people worldwide have suffered from dementia, and this number will reach 150 million by the middle leaf of the 21st century. At present, the global cost of dementia has reached 818 billion dollars, and a case of dementia occurs about every 3 seconds in the world [38]. Targeting at investigating the pathogenesis of AD and exploring potential therapeutic drugs, “Brain Plan” has been carried out in many countries. Although new drugs such as Aducanumab and Sodium oligomannate (GV-971), targeting at the classical amyloid beta or the Brian-Gut axis, have been approved to be used in treatment of AD, their clinic effect are controversial and more clinic trails are required.

STZ is a glucosamine-nitrosourea and DNA alkylating reagent that synthesized by Streptomycetes achromogenes. STZ could result in insulin resistance, and has been widely used to establish animal models of diabetes and AD [39]. It has been reported that lateral ventricle injection of STZ could induce AD-like performance in rats [40] or mice [42–46], as indicated by a significant cognitive impairment and increased Aβ1−42 accumulation and hyper-phosphorylated tau in the hippocampus[41], together with increased glial activation or decreased expression of synapse-associated protein [41–45]. In our previous study, STZ was administrated via bilateral hippocampal injection, and the mice presented impaired learning and memory ability, together with insulin resistance and hyper-activated microglial and alternative synaptic plasticity in the hippocampus [46]. Consistently, in the present study, our results showed that lateral ventricle injection of STZ mice could induce AD-like behavioral performance in mice, as indicated by the decreased frequency, duration, and moving distance in the center area and reduced rearing frequency in the OFT, increased immobility in the FST, and the decreased preference index of novel object or arm in the NOR or Y maze. Unsurprisingly, a positive correlation was found between the decreased activity in the OFT and the decreased learning and memory ability in the NOR. These results indicated again the efficacy, repeatability, and reliability of STZ in establishing AD-like animal models. Moreover, in the present study, mice treated with BMSC-exos via lateral ventricle injection, but not caudal vein injection, presented increased activity and enhanced rearing frequency in the OFT, together with the increased preference index of novel object in the NOR. These results indicated that lateral ventricle injection of BMSC-exos could reverse the STZ-injected AD-like behaviors in mice with a task-specific manner. We could not interpret exactly the reason of the different effect of BMSC-exos between the two delivery routes, although the immunofluorescence staining results showed that the BMSC-exos labeled with PKH26 could be detected in the hippocampus when given by lateral ventricle injection, but not caudal vein injection. The method of administration of exosomes injected into the lateral ventricle has shown good therapeutic effects in existing studies [47, 48]. However, there are many factors influencing whether to pass the "dynamic protective wall" of the blood-brain barrier [49], and it is still controversial whether the exosomes injected by the caudal vein administration route can smoothly pass through the blood-brain barrier and enter the central system. Thus, the discrepancy might be, at least partly, ascribed to the ability to cross successfully the blood-brain barrier and the limited injection volume.
The activation and proliferation of central glial cells is one of the most significant pathological features of AD. Microglia are considered to be resident macrophages of the central system, which are in close contact with neurons and astrocytes [50, 51]. Microglia play two roles in the entire pathological process of AD, from the "cleaner" in the early stage of AD to the "accomplice" in the later stage [52]. From a morphological point of view, round microglia have a phagocytic function, and studies have pointed out it. The microglia in the drug treatment group recovered from the activated irregular polygon shape to a round shape, and the Aβ contained in their phagosomes was twice that of activated microglia [53]. After the body is injured, microglia take the lead to respond, and then astrocytes begin to respond [54]. After activation, astrocytes will release inflammatory factors just like activated microglia, which will further aggravate neuroinflammation. In our present study, the number of activated microglia and astrocytes in the hippocampus of the model group mice increased significantly. After treatment with BMSC-exos, the number of activated microglia and astrocytes decreased significantly in the Lv group, but not the Cv group, and the number of round microglia in the Lv group was also increased significantly and returned to the normal level in the comparison with model group. Moreover, results of Western blot showed the similar results with the immunofluorescence staining, and the expression of GFAP and Iba1 was positively correlated. These results indicated again the important role of glial activation in the pathogenesis of AD, and it might be a potential target of BMSC-exos in relieving AD-like performance.

As mentioned earlier, the activation of glial cells will strengthen the inflammatory response, and the interaction between glial cells and inflammatory factors will form a vicious circle, and will trigger a series of malignant involvement, such as promoting the accumulation of Aβ and phosphorylation of Tau protein [9–13], which are two characteristic neuropathological hallmarks of AD. Consistently, in the present study, the mRNA and protein expression levels of IL-1β, IL-6, and TNF-α in the hippocampus of the model mice were up-regulated, together with an increased protein expression of BACE, Aβ1−42, and p-Tau. Moreover, apart from the expected positive correlation between the inflammatory cytokines, the expression of IL-1β was also found positively correlated to the expression of GFAP, Iba1, BACE, Aβ1−42 and p-Tau. Additionally, the preference index of novel object in the NOR was negatively correlated to the expression of GFAP, IBA1 and IL-1β in the hippocampus. These findings suggested again the network hypothesis of AD which proposes that AD is a complex and multifactorial disease with the compromised functionality of relevant neural networks underlying the development of AD symptomatology. After BMSC-exos treatment via intracerebroventricular injection, the levels of inflammatory factors were significantly down-regulated, and the expression levels of BACE, Aβ1−42 and p-Tau proteins were also significantly reduced. These results indicated that BMSC-exos may alleviate the central nervous system inflammation and improve the typical neuropathological features of AD in mice injected by STZ.

Synaptic plasticity is the material basis for regulating learning and memory functions [55]. It has been long demonstrated that the expression levels of synapse-related proteins such as Synaptotagmin-1 and Synapsin-1 are significantly reduced in the hippocampal tissues of postmortem AD patients and animal models [56–59]. BDNF is one of the important regulators that highlight plasticity [60], can provide nutritional support for neurons and improve learning and memory impairment by reversing neuronal loss.
In our previous studies, imbalanced expression of BDNF in the hippocampus or prefrontal cortex and its related adaptive change of synaptic plasticity including decreased expression of Synaptotagmin-1, Synapsin-1, and PSG95 have been demonstrated to be associated with impairment of learning and memory or depression-like behaviors in animal models with depression, SCH, NAFLD or AD [62–64]. In the present study, the expression of BDNF, Synaptotagmin-1, and Synapsin-1 was decreased in the hippocampus of STZ-intracerebroventricular injected mice, suggesting again the important role of BDNF and its associated synaptic proteins in the sustainment of learning and memory. Moreover, the expression of BDNF was positively correlated to the preference index of novel object in the NOR and the hippocampal expression of Syn1, but negatively correlated to the expression of GFAP, Iba1, IL-1β, IL-6, TNF-α, Aβ1−42, and p-Tau. These results indicated that BDNF might be a key molecular linking the relevant multiple pathogenesis underlying the development of AD. However, the decreased expression of BDNF in the AD mice model was reversed by lateral ventricle administration of BMSC-exos. Although there was no significant difference between mice in the model group and the Lv group, results of IF showed that the expression of DCX, a new-born neuron marker, was significantly increased in the hippocampus of mice in the Lv group than that in the model group. These results suggest that BMSC-exo may regulate the prominent plasticity of the hippocampus by up-regulating the expression of BDNF and promoting the neuronal regeneration.

Conclusions

In conclusion, our results indicated that lateral ventricle administration of BMSC-exos could improve the AD-like behaviors in mice, the mechanism of which might be associated with the regulation of glial activation and its associated neuroinflammation and BDNF-related neuropathological changes in the hippocampus. The summary sketch was shown in Fig. 11.

Abbreviations

AD
Alzheimer's disease; Aβ:β-amyloid; ACHE:acetylcholin esterase; BDNF:Brain-derived neurotrophic factor; IL-1β:Interleukin 1β; IL-6:Interleukin 6; TNF-α:Tumor necrosis factor; BMSCs:Bone marrow mesenchymal stem cells; BMSC-exos:Exosomes derived from bone marrow mesenchymal stem cells; p-Tau:phosphorylated Tau protein; GFAP:Glial fibrillary acidic protein; IBA1:Ionized calcium binding adapter molecule 1; DCX:Recombinant Doublecortin; CD44:Cluster of differentiation 44; CD45:Cluster of differentiation 45; CD11b:Cluster of differentiation 11b; CD63:Cluster of differentiation 63; HSP70:Heat shock protein 70; TSG101:Tumor susceptibility gene 101 protein; OFT:Open field test; EPM:Elevated plus maze test; Y-maze:Y maze test; NOR:Novel object recognition test; TST:Tail suspension test.

Declarations

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**Authors’ contributions**

Liu Sen and Fan Min conducted most of the experiments, analyzed the data and wrote the manuscript. Xu Jingxian assisted in cell culture, and Xia Qingrong and Qi congcong modified the picture. This study was designed by Liu Sen and Ge Jinfang. Ge Jinfang conceived the study and revised the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

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

**Ethics approval and consent to participate**

The animal study was reviewed and approved by the Animal Experimentation Ethics Committee of Anhui Medical University. Written informed consent was obtained from all individuals who were included in the study.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interest.

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**Figures**
Figure 1

Experimental process. STZ, streptozotocin; ACSF, artificial cerebrospinal fluid; OFT, open field test; NOR, novel object recognition test; Y-maze, Y maze test; TST, tail suspension test.
Figure 2

extract, culture, and sorting of BMSCs, and identification and label of BMSC-exos. A: Bone marrow primary adherent cells before sorting. B: CD44+/CD45-/CD11b- BMSCs were sorted out by flow sorting, BMSCs grew into fibrous and colony-like growth, scale bar 100μm. C: Exosome morphology under transmission electron microscope. D: Western blot photos of exosome markers. E: Fluorescence imaging of exosome labeled with PKH26.
Figure 3

Exosomes injection site and its trace. A: Nissl staining shows that the catheter is accurately positioned to the lateral ventricle. B: PKH26-labeled red fluorescent exosome particles are observed in the hippocampus of Lv group. C: Partial enlarged view of exosomes labeled with PKH26.
Lateral ventricle administration of BMSC-exos improved the AD-like behaviors induced by intracerebroventricular injection of STZ in mice. A: total ambulatory distance in the OFT; B: number of line crossed in the OFT. C: frequency to the central region in the OFT. D: moving distance in the central region in the OFT. E: number of rearing in the OFT. F: moving distance in the closed arm in the EPM. G: preference index of the novel object in the NOR. H: preference index of the novel arm in the Y-maze. I:
Immobility time in the TST. J: Representative trajectories in OFT, EPM, Y-maze and NOR. K: The duration in the centre in the OFT was positively correlated to the preference of novel object in the NOR. L: The distance in the centre in the OFT was positively correlated to the preference of novel object in the NOR. M: The number of rearing in the OFT was positively correlated to the preference of novel object in the NOR. Data are presented as means ± SEM, with n=6 in each group (*p < 0.05, **p < 0.01)

**Figure 5**

Lateral ventricle administration of BMSC-exos inhibited the proliferation and activation of microglia in the hippocampus of mice injected with STZ. A: Fluorescence detection of microglia marker IBA1 in hippocampus B: Quantification of the number of IBA1 positive cells. C: Microglia in different states (left: Resting state; right: activated state); D: the ratio of activated microglia to the total number of microglia. Data are presented as means ± SEM, with n=3 in each group (*p < 0.05, **p < 0.01)
Figure 6

Lateral ventricle administration of BMSC-exos inhibited the activation of astrocytes in the hippocampus of mice injected with STZ. A: Fluorescence detection of GFAP, a marker of activated astrocytes in hippocampus; B: quantification of the number of GFAP-positive cells. C: Western Blot photos of IBA1 and GFAP; D: IBA1 and GFAP protein expression levels quantification. Data are presented as means ± SEM, with n=3 in each group (*p < 0.05, **p < 0.01)
Figure 7

Lateral ventricle administration of BMSC-exos reduced the expression levels of IL-1β, IL-6 and TNF-α, Aβ, and p-Tau in the hippocampus of mice injected with STZ. A: Typical Western Blot photos of IL-1β, IL-6 and TNF-α; B. Quantitative analysis of the protein expression of IL-1β, IL-6 and TNF-α. C: Quantitative analysis of the mRNA expression of IL-1β, IL-6 and TNF-α. D: Typical Western blot photographs of Aβ1-42, BACE and p-Tau (ser396). E: Quantitative analysis of the protein expression of Aβ1-42, BACE, and p-Tau.
(ser396) expression level. Data are presented as means ± SEM, with n=3 in each group (*p < 0.05, **p < 0.01)

Figure 8

Correlation analysis between the hippocampal expression of GFAP, IBA1, IL-1β, IL-6, TNF-α, BACE, Aβ1-42, p-Tau and the preference index of novel object in the NOR. A: Expression of IL-1β was positively correlated to IBA1. B: Expression of IL-1β was positively correlated to IL-6. C: Expression of IL-1β was positively correlated to TNFα. D: The preference index of novel object was negatively correlated to the expression of GFAP. E: The preference index of novel object was negatively correlated to expression of IBA1. F: The preference index of novel object was negatively correlated to the expression of IL-1β. G: Expression of BACE was positively correlated to Aβ1-42. H: Expression of p-Tau was positively correlated to Aβ1-42. I: Expression of Aβ1-42 was negatively correlated to the preference index of novel object.
Lateral ventricle administration of BMSC-exos promoted neuron regeneration and the expression of BDNF in the hippocampus of mice injected with STZ. A: Fluorescence detection of the hippocampal neonatal neuron marker DCX; B: Statistics of the number of DCX positive cells. A: Western blot photos of Synaptotagmin-1, Synapsin-1 and BDNF; B: Quantification of Synaptotagmin-1, Synapsin-1 and BDNF expression levels. Data are presented as means ± SEM, with n=3 in each group (*p < 0.05, **p < 0.01)

**Figure 9**
Figure 10

The Pearson's correlation test between the hippocampus expression of GFAP, IBA1, IL-1β, IL-6, TNF-α, BACE, Aβ1-42, p-Tau, the number of rearing in the OFT and the preference index of novel object in the NOR. A: The number of rearing was positively correlated to the expression of BDNF. B: The preference of novel object was positively correlated to the expression of BDNF. C: Expression of Syt-1 was positively correlated to BDNF. D: Expression of IBA1 was positively correlated to BDNF. E: Expression of IL-1β was positively correlated to BDNF. F: Expression of Aβ1-42 was positively correlated to BDNF.
Figure 11

Exosomes derived from bone marrow mesenchymal stem cells reduce neuroinflammation and synaptic damage by inhibiting the activation of glial cells in the hippocampus.