Journal of Integrative Neuroscience

J. Integr. Neurosci. **2023**; 22(6): 144
https://doi.org/10.31083/j.jin2206144

Original Research

Marrow Mesenchymal Stem Cell-Derived Exosomes Upregulate Astrocytic Glutamate Transporter-1 Expression via miR-124/mTOR Pathway against Oxygen-Glucose Deprivation/Reperfusion Injury

Weiyi Huang[1,*,†], Yuansheng Fan[1,†], Chen Jiang[1], Jiantong Jiao[1], Wei Ji[1], Hui Huang[1], Junfei Shao[1,*]

[1]Department of Neurosurgery, The Affiliated Wuxi People's Hospital of Nanjing Medical University, Wuxi People's Hospital, Wuxi Medical Center, Nanjing Medical University, 214023 Wuxi, Jiangsu, China

*Correspondence: weiy_huang@126.com (Weiyi Huang); wxbrain@163.com (Junfei Shao)

[†]These authors contributed equally.

Academic Editor: Gernot Riedel

Submitted: 4 May 2023 Revised: 11 July 2023 Accepted: 13 July 2023 Published: 23 October 2023

Abstract

Background: Experimental investigations have reported the efficacy of marrow mesenchymal stem cell-derived exosomes (MSC-Exos) for the treatment of ischemic stroke. The therapeutic mechanism, however, is still unknown. The purpose of the study is to show whether MSC-Exos increases astrocytic glutamate transporter-1 (GLT-1) expression in response to ischemic stroke and to investigate further mechanisms. **Methods and Results**: An *in vitro* ischemia model (oxygen-glucose deprivation/reperfusion, OGD/R) was used. MSC-Exos was identified by Western blot (WB) and transmission electron microscopy (TEM). To further investigate the mechanism, MSC-Exos, miR-124 inhibitor, and mimics, and a mTOR pathway inhibitor (rapamycin, Rap) were used. The interaction between GLT-1 and miR-124 was analyzed by luciferase reporter assay. The GLT-1 RNA expression and miR-124 was assessed by quantitative real-time polymerase chain reaction (qRTPCR). The protein expressions of GLT-1, S6, and pS6 were detected by WB. Results demonstrated that MSC-Exos successfully inhibited the decrease of GLT-1 and miR-124 expression and the increase of pS6 expression in astrocytes after OGD/R. miR-124 inhibitor suppressed the effect of MSC-Exos on GLT-1 upregulation after OGD/R. Rapamycin notably decreased pS6 expression with significantly higher GLT-1 expression in astrocytes injured by OGD/R. Luciferase activity of the reporter harboring the wild-type or mutant GLT-1 3′UTR was not inhibited by miR-124 mimics. Further results showed that the inhibiting effect of MSC-Exos on pS6 expression and promoting effect of MSC-Exos on GLT-1 expression could be reversed by miR-124 inhibitor after OGD/R; meanwhile, the above conditions could be reversed again by rapamycin. **Conclusions**: Results show that miR-124 and the mTOR pathway are involved in regulation of MSC-Exos on GLT-1 expression in astrocytes injured by OGD/R. miR-124 does not directly target GLT-1. MSC-Exos upregulates GLT-1 expression via the miR-124/mTOR pathway in astrocytes injured by OGD/R.

Keywords: ischemic stroke; marrow mesenchymal stem cells; exosome; oxygen-glucose deprivation/reperfusion; GLT-1

1. Introduction

The effectiveness of marrow mesenchymal stem cells (MSCs) in the treatment of ischemic stroke has been demonstrated by both experimental studies and early clinical trials [1,2]. Paracrine action is the main way MSCs exert therapeutic effects. One of the key factors for paracrine effects is marrow mesenchymal stem cell-derived exosome (MSC-Exo). It has multiple characteristics such as small size, high stability, easy access, low immunogenicity, almost no graft reaction, and easily passes through the blood-brain barrier. MSC-Exos have become a research focus for the therapy of ischemic stroke [3,4]. Nevertheless, therapeutic mechanisms of MSC-Exos in ischemic stroke should be further explored.

Studies have investigated that MSC-Exos protect against ischemic stroke by reducing microglia accumulation, increasing angiogenesis, inducing brain remodeling, and so on [5,6]. But, whether MSC-Exos could inhibit excitotoxic injury has not been reported. After ischemic stroke, glutamate transporters expression decreases, which results in excitotoxic injury with associated uptake disorders and excessive accumulation of extracellular glutamate [7]. Glutamate transporters, which have five subtypes including excitatory amino acid transporters (EAAT) 1-EAAT5, are regarded as the only way for nerve cells to take up extracellular glutamate [8]. EAAT2 (GLT-1, glutamate transporter-1), which is a therapeutic target for inhibition of excitotoxic injury, accounts for about 90% of the total intake of extracellular glutamate. Studies have shown that the upregulation of GLT-1 effectively attenuates the brain infarct area and improves neurological deficits in ischemic stroke [9,10]. However, whether MSC-Exos upregulates GLT-1 expression after ischemic stroke to exert therapeutic effects has not been reported and further mechanisms need to be demonstrated.

MSC-Exos contain many biological components such as proteins, DNA, mRNAs, and miRNAs [11]. MSC-Exos

could exert therapeutic effects by regulating miRNA expression against ischemic stroke [12]. It has been reported that miR-124 might promote GLT-1 expression in normal cultured astrocytes and prevent GLT-1 from being downregulated in a model of amyotrophic lateral sclerosis [13]. Hence, it is concluded that MSC-Exos might affect GLT-1 expression by regulating miR-124. The mTOR pathway, which is regarded as a target of therapy for ischemic stroke, plays a significant role in its pathogenic progression [14]. It has been shown that insulin promotes the downregulated GLT-1 expression induced by Abeta through the mTOR pathway [15]. However, there is no evidence as to whether MSC-Exos influences GLT-1 expression via the mTOR pathway in ischemic stroke. Meanwhile, some studies and our previous research have confirmed that miR-124 regulates nerve cell function through the mTOR pathway [16,17]. Thus, it is further hypothesized that miR-124 might participate in protecting against ischemic stroke by regulation of MSC-Exos on GLT-1 expression via the mTOR pathway.

In this study, an *in vitro* model of ischemic stroke (oxygen-glucose deprivation/reperfusion, OGD/R) was used. Meanwhile, MSC-Exos, miR-124 inhibitor and mimics and a mTOR pathway inhibitor were used to further explore the mechanisms. Results demonstrated that MSC-Exos upregulated GLT-1 expression via the miR-124/mTOR pathway against ischemic stroke, which might present new experimental evidence for the therapeutic mechanisms of MSC-Exos for treatment of ischemic stroke.

2. Materials and Methods

2.1 Cell Culture

The preparation and identification of MSCs and astrocytes were performed according to a previous study [15]. The MSCs and astrocytes had been tested for mycoplasma contamination. For MSCs, mononuclear cells from adult Wistar rats weighing 120–180 g were removed from their femurs and tibias. MSCs were then separated by centrifuging cells at 900 g for 20 min at a density of 1.073 g/mL. Cells were then grown (2×10^5 cells/cm^2) in 5 mL of DMEM/F12 (1:1) with 10 % fetal bovine serum (FBS) and 1% penicillin-streptomycin (100 U/mL), after being washed twice then centrifuged (400 g, 5 min). After 72 hours, non-adherent cells were removed, and new medium used. At 80% confluence, cells were trypsinized, collected, and expanded. In prior investigation, flow cytometry (Beckman coulter, Brea, CA, USA) was used to identify MSCs [18]. The 3rd–8th generations of MSCs were used for the follow-up experiments.

For astrocytes, one day old Wistar rats had their brains removed to provide primary cortical astrocytes. Firstly, the cerebral cortices were dissected, then meninges stripped. The 0.125% trypsin was used to treat the above cerebral cortex for 15 min at 37 °C. Then, cells of the centrifugated deposit were resuspended and seeded into 75 cm^2 plastic flasks coated with poly-L-lysine at a density of 2×10^5 cells/cm^2. The cells were grown in the medium which was changed every three days. 10–14 days later, to get rid of the oligodendrocytes and microglia that were just lightly adhered, flasks were capped and vibrated at 220 rpm overnight. The cells on the flasks were trypsinized and moved to other flasks and identified as astrocytes by immunofluorescence staining of glial fibrillary acidic protein (GFAP) as described in a previous study [18].

2.2 Exosome Isolation

MSCs were inoculated in 75 cm^2 flasks in DMEM/F12 containing 10% FBS. When covering about 80% of the bottom of the flask, cells were transferred to DMEM/F12 containing 10% FBS without exosomes. After 48 h, the supernatant was transferred into a tube and centrifuged at 3000 rpm (15min) to remove cells and cell debris. The ExoQuick-TC exosome precipitation solution (10 mL medium added to 2 mL ExoQuick-TC, EXOTC10A-1, System Biosciences, Palo Alto, CA, USA) was used to treat the supernatant. The mixed solution was incubated at 4 °C overnight, and centrifuged at 1500 rpm (30 min) to settle exosomes to the bottom. After removing the supernatant, the mixed solution was again centrifuged at 1500 rpm (5 min). After removing the supernatant, exosomes were diluted with PBS at a certain concentration and stored at 4 °C for use within one week.

2.3 Exosome Identification

The total protein concentrations of exosomes, which represented concentrations of MSC-Exos, were detected by the bicinchoninic acid (BCA) protocol (P0010S, Beyotime, Shanghai, China). Exosome morphology was examined by Transmission Electron Microscopy (TEM) (Hitachi, Tokyo, Japan). In brief, glutaraldehyde was used to fix the purified exosome solution which had been diluted to 500 µg/L. The copper network was then treated with 20 µL of the fixed solution and stained for 5 min with a 3% phosphotungstic acid solution. Exosome ultrastructure was examined by TEM after drying. The marker proteins CD63, CD9, and CD81 of exosomes were analyzed by Western Blot (WB).

2.4 Treatment of Astrocytes Injured by OGD/R

Astrocytes were washed before being cultured in oxygen-glucose deprivation (OGD) medium (glucose-free DMEM) for 6 hours at 37 °C in an anaerobic chamber with a combination of 95% N_2 and 5% CO_2 (OGD group). OGD 6 h later, cells were rinsed twice then cultured in complete medium (10% FBS-containing DMEM/F12, OGD/R group), complete medium with different concentrations of MSC-Exos medium (OGD/R + 0 µg/mL, OGD/R + 25 µg/mL, OGD/R + 50 µg/mL, OGD/R + 100 µg/mL) and complete medium with added mTOR inhibitor (rapamycin, OGD/R + Rap group) for 24 h. To further investigate the regulation of MSC-Exos on GLT-1, miR-124 mimics (mim-

ics group), miR-124 inhibitor (inhibitor group), or respectively negative controls (Mnc group and Inc group) were transfected into astrocytes. OGD 6 h later, astrocytes were cultured in either complete medium, MSC-Exos medium, or complete medium supplemented with rapamycin. Astrocytes without OGD/R in the normal condition were considered the control group (Control group).

2.5 Transfection

Lipofectamine 2000 (11668500, Invitrogen, Carlsbad, CA, USA) was utilized to transfect the plasmids and miRNAs (GenePharm, Suzhou, Jiangsu, China) following the manufacturer's instructions. Cells were cultivated in a 6-well plate at a density of 1×10^5 cells per well until they were 80% confluent. A 1:1 dilution of 3 µL of miRNA in 300 µL of Opti-MEM and Lipofectamine 2000 was performed. Astrocytes were treated with the miRNA-lipid combination and incubated for 48 hours at 37 °C. MiR-124 mimics, inhibitor, or negative vectors were transfected into the astrocytes, which were identified by quantitative polymerase chain reaction (qPCR) (Bioer, Hangzhou, Zhejiang, China) and immunofluorescence (Leica, Wetzlar, Germany).

2.6 RNA Isolation and Real-Time Polymerase Chain Reaction (qRTPCR)

Total RNA was extracted by Trizol Reagent (15596026, Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. The ABI Step One Plus Real-Time PCR System (Applied Biosystems, Grand Island, NY, USA) was used to measure the GLT-1 mRNA expression. The endogenous control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was taken into consideration. Three duplicates of each experiment were carried out. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative mRNA expression. The forward (F) and reverse (R) primers were produced by Sangon Biotech (Shanghai, China):

GLT-1-F:5′-CCAAGCTTGGATCACTGCCCTGG-3′,

GLT-1-R: 5′-CCAGCCCCAAAAGAGTCACCCA CAA-3′

GAPDH-F: 5′-CCCCCAATGTATCCGTTGTG-3′,

GAPDH-R:5′-TAGCCCAGGATGCCCTTTAGT-3′;

MiR-124 primers and the reference snoRNA202 were used to transform the miRNA to cDNA with a TaqMan miRNA reverse transcription kit (4366596, Applied Biosystems). SYBR Green (4309155, Invitrogen, Carlsbad, CA, USA) was used to assess the amount of miR-124. An artificial miR-124 oligo was used to create a standard curve (5′-UAAGGCACGCGGUGAAUGCC-3′).

2.7 Protein Isolation and Western Blotting

Cell lysis and protein extraction kits were used to process astrocytes and MSC-Exos samples. The BCA protein detection kit (P0010S, Beyotime, Shanghai, China) was used to measure the protein concentration. Samples were subjected to Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (3010040001, Millipore Corporation, Billerica, MA, USA). Membranes were incubated overnight at 4 °C with anti-CD9 (diluted 1:1000, #98327, Cell Signaling Technology (CST), Danvers, MA, USA), anti-GLT-1 (diluted 1:1000, #3838, CST, Danvers, MA, USA), anti-S6 (diluted 1:1000, #2217, CST, Danvers, MA, USA), anti-pS6 (diluted 1:1000, #2215, CST, Danvers, MA, USA), anti-GAPDH (diluted 1:1000, #5174, CST, Danvers, MA, USA), anti-CD63 (diluted 1:1000, sc-5275, Santa Cruz Biotechnology, Dallas, TX, USA) and anti-CD81 (diluted 1:1000, sc-166029, Santa Cruz Biotechnology, Dallas, TX, USA). After three rounds of washing, the membranes were incubated for 1 h at room temperature with anti-mouse (diluted 1:5000, SA00001-1, proteintech, Wuhan, Hubei, China) or anti-rabbit (diluted 1:5000, SA00001-2, proteintech, Wuhan, Hubei, China) secondary antibodies conjugated with horseradish peroxidase. An improved chemiluminescence detection agent was used to develop protein blots. Quantity One, 1-D analysis software (Version 4.4, Bio-Rad, Hercules, CA, USA) was used to analyze data, which were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Each immunoblot was performed three times.

2.8 Luciferase Reporter Assay

The GLT-1 mRNA 3′UTR containing miR-124 binding sites was multiplied from cDNA and added into a pmirGLO vector (E1330, Promega, Madison, WI, USA) to create pmirGLO-GLT-1 3′UTR-wild type (WT GLT-1) by GenePharma. Meanwhile, the pmirGLO-GLT-1 3′UTR-mutant (MUT GLT-1) with mutation of predicted miR-124 binding sites was made by the QuikChange Site-directed Mutagenesis kit (200518, Agilent Technologies, Inc., Santa Clara, CA, USA). Then, luciferase reporter plasmids were co-transfected into HEK293T cells containing GLT-1 3′UTR and miR-124 mimics or Mnc. The firefly and renilla luciferase activity was measured by a dual luciferase reporter assay system (E1910, Promega, Madison, WI, USA) 2 days later. Renilla luciferase was used as a normalizing factor to calculate relative luciferase activity.

2.9 Statistical Analysis

GraphPad Prism (Version 9, GraphPad Software Inc., Boston, MA, USA) was used to conduct statistical analysis, and the data analyzed in this study was displayed as the mean ± standard deviation. The differences between various groups were analyzed by a one-way analysis of variance, and the Student's *t*-test was employed to compare the differences between the two groups. The Bonferroni method was used to examine multiple comparisons. A p-value < 0.05 was considered significant.

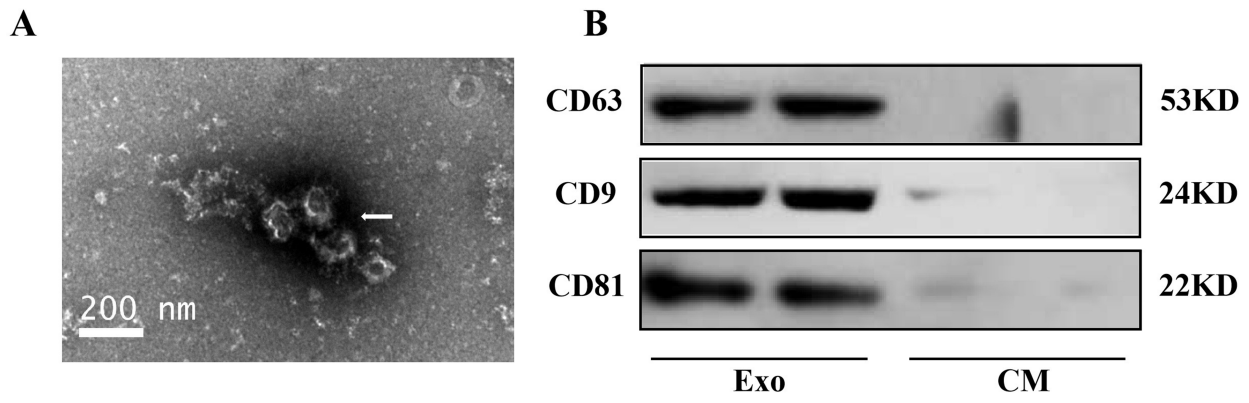

Fig. 1. Exosomes' ultrastructure was detected by TEM and marker proteins were analyzed by WB. (A) The morphology of MSC-Exos (scale bar = 200 nm). Arrow points to the site of MSC-Exos. (B) WB detected the expression of CD63, CD9 and CD81, which were the marker proteins of MSC-Exos. MSC-CM (conditioned medium) was used as the control group. TEM, transmission electron microscopy; WB, western blot; MSC-Exos, marrow mesenchymal stem cell-derived exosomes.

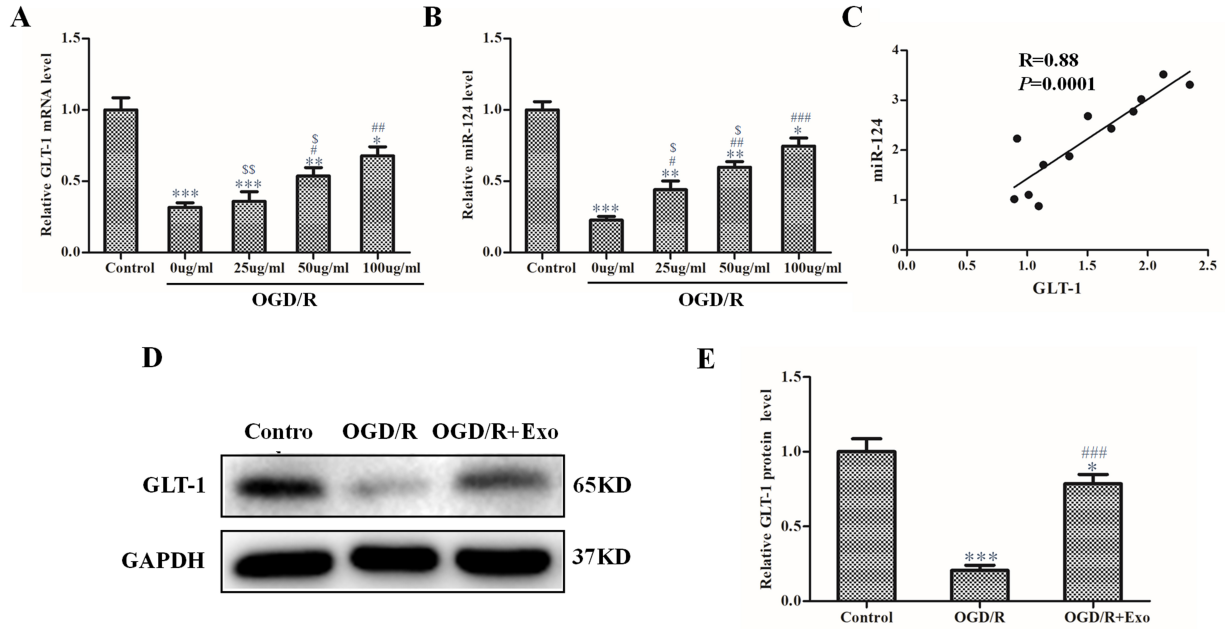

Fig. 2. MSC-Exos regulated GLT-1 and miR-124 expression in astrocytes after OGD/R. (A,B) The effects of different concentrations of MSC-Exos on GLT-1 mRNA and miR-124 expressions after OGD/R. (C) A positive association between the GLT-1 mRNA and miR-124, correlation analysis coefficient $R = 0.88$, $p = 0.0001$. (D,E) The effects of MSC-Exos on GLT-1 protein expression in astrocytes after OGD/R. Compared with control group, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; compared with 0 μg/mL MSC-Exos group (OGD/R group), # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$; compared with 100 μg/mL MSC-Exos group (OGD/R + Exo group); $ $p < 0.05$, $$ $p < 0.01$. As a ratio to the internal, the relative protein expression was displayed (n = 3). GLT-1, glutamate transporter-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; OGD/R, oxygen-glucose deprivation/reperfusion.

3. Results

3.1 MSC-Exos Identification

It could be clearly seen that the extracts were membranous vesicles, round or oval, with clear edges and double lipid membranes detected by the TEM (Fig. 1A). WB indicated that exosome-specific proteins, CD63, CD9, and CD81, were positive (Fig. 1B). The above results confirmed that the extracts were MSC-Exos.

3.2 MSC-Exos Regulated GLT-1 and miR-124 Expression in Astrocytes Injured by OGD/R

After OGD/R, expressions of the GLT-1 mRNA and miR-124 in OGD/R group (0 μg/mL MSC-Exos) decreased significantly compared to control ($p < 0.001$) (Fig. 2A,B). As the concentration of MSC-Exos increased, the GLT-1 mRNA and miR-124 expressions showed gradual upregulation. Pearson's correlation analysis revealed a positive

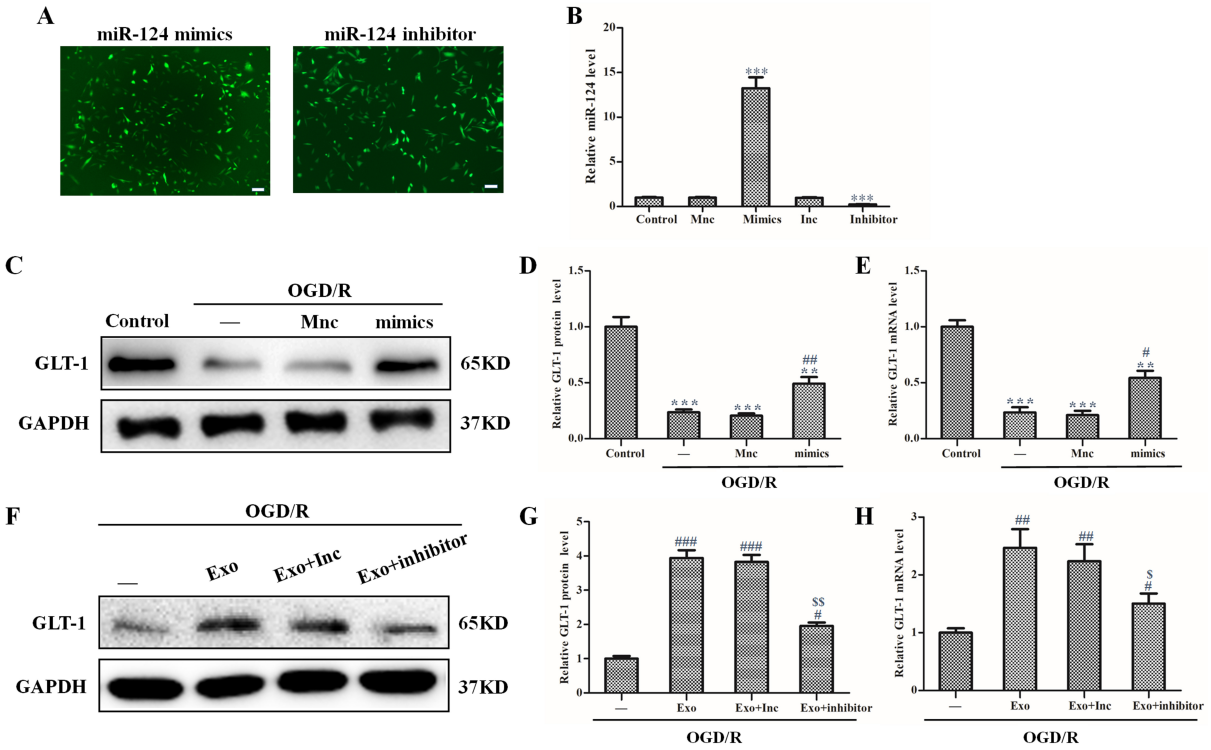

Fig. 3. MSC-Exos upregulated GLT-1 expression through miR-124 in astrocytes after OGD/R. (A) Astrocytes transfected with miR-124 mimics or inhibitor plasmids were detected by immunofluorescence (bar = 200 μm). (B) miR-124 mimics or inhibitor were effectively transfected into astrocytes. miR-124 expression was detected by quantitative real-time polymerase chain reaction (qRTPCR). (C–E) The impact of miR-124 mimics on GLT-1 expression in astrocytes injured by OGD/R. (F–H) miR-124 inhibitor suppressed the upregulation of MSC-Exos on GLT-1 in astrocytes after OGD/R. GLT-1 expression was detected by qRTPCR or WB. Compared with control group, ** $p < 0.01$, *** $p < 0.001$; compared with OGD/R group, # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$; compared with OGD/R + Exo group, \$ $p < 0.05$, \$\$ $p < 0.01$. As a ratio to the internal, the relative protein expression was displayed (n = 3).

association between the GLT-1 mRNA and miR-124 expressions (R = 0.88, $p = 0.0001$) (Fig. 2C). The GLT-1 mRNA and miR-124 expressions were both highest under the 100μg/mL MSC-Exos (Exo group) compared with other conditions ($p < 0.05$) (Fig. 2A,B). In comparison to the OGD/R group, GLT-1 protein expression was dramatically increased under 100 μg/mL MSC-Exos ($p < 0.001$) (Fig. 2D,E), which was used for follow-up experiments.

3.3 MSC-Exos Upregulated GLT-1 Expression through miR-124 in Astrocytes Injured by OGD/R

Astrocytes transfected with miR-124 mimics or inhibitor showed green with immunofluorescence. Compared with the control group, miR-124 expression significantly elevated in the mimics group and significantly decreased in the inhibitor group ($p < 0.001$). miR-124 expression was unaffected in the Mnc or Inc group, compared with the control group (Fig. 3A,B). When compared with the control group, OGD/R induced notably lower GLT-1 mRNA and protein expression in astrocytes ($p < 0.001$). However, downregulation of GLT-1 mRNA and protein expression could be reversed by miR-124 mimics. When compared with the OGD/R group, miR-124 mimics significantly increased GLT-1 mRNA and protein expression ($p < 0.05$) (Fig. 3C–E). Additionally, the upregulation of MSC-Exos on GLT-1 was significantly suppressed by miR-124 inhibitor. The OGD/R + Exo + inhibitor (miR-124 inhibitor) group had lower GLT-1 mRNA and protein expression when compared with the OGD/R + Exo group ($p < 0.05$) (Fig. 3F–H).

3.4 MSC-Exos Upregulated GLT-1 Expression via the mTOR Pathway in Astrocytes Injured by OGD/R

In comparison to the control group, OGD/R notably increased the pS6 (an indicator of mTOR activity) protein expression in astrocytes ($p < 0.001$). Meanwhile, in comparison to the OGD/R group, MSC-Exos significantly downregulated the overexpression of pS6 and promote GLT-1 expression ($p < 0.05$) (Fig. 4A,B; Fig. 2D,E). Additionally, rapamycin also suppressed the overexpression of pS6 and increased GLT-1 expression in astrocytes injured by OGD/R ($p < 0.05$) (Fig. 4C–E). The above findings indirectly confirmed that MSC-Exos upregulated GLT-1 expression via the mTOR pathway in astrocytes injured by OGD/R.

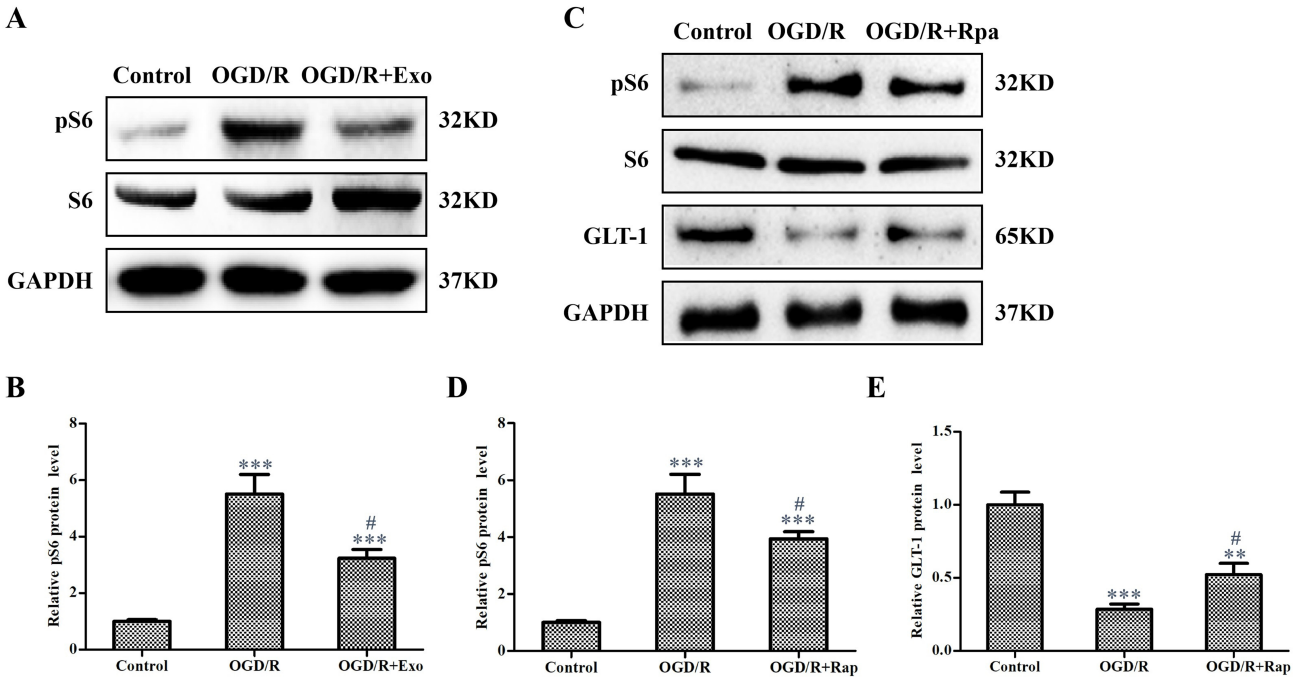

Fig. 4. MSC-Exos upregulated GLT-1 expression through the mTOR pathway in astrocytes after OGD/R. (A,B) MSC-Exos regulated the mTOR pathway (pS6 and S6) protein expression in astrocytes after OGD/R. (C–E) The effects of mTOR pathway inhibitor, rapamycin, on the pS6, S6 and GLT-1 protein expression in astrocytes after OGD/R. Compared with control group, ** $p < 0.01$, *** $p < 0.001$; compared with OGD/R group, # $p < 0.05$. As a ratio to the internal, the relative protein expression was displayed (n = 3).

3.5 MSC-Exos Upregulated GLT-1 Expression via the miR-124/mTOR Pathway in Astrocytes Injured by OGD/R

The luciferase reporter assay showed that the co-transfection of miR-124 mimics did not affect relative luciferase activities of the WT or MUT GLT-1 3′UTR reporter when compared with mimics negative control (NC) ($p >$ 0.05), which revealed that miR-124 did not directly target GLT-1 (Fig. 5A).

Notably, miR-124 mimics reversed the upregulation of pS6 expression in astrocytes injured by OGD/R. Compared with the OGD/R group, the OGD/R + mimics group showed a significant decrease in pS6 protein expression ($p < 0.05$), while the OGD/R + inhibitor group showed a significant increase in pS6 protein expression ($p < 0.05$). Whereas the OGD/R + Mnc or OGD/R + Inc groups showed no significant effect on pS6 expression (Fig. 5B,C). These findings demonstrate that miR-124 regulated the mTOR pathway in astrocytes injured by OGD/R.

When compared with the OGD/R + Exo group, the OGD/R + Exo + miR-124-(miR-124 inhibitor) group showed significantly higher levels of pS6 protein expression and significantly lower levels of GLT-1 protein expression ($p < 0.001$). In comparison to the OGD/R + Exo + miR-124- group, the pS6 protein expression in the OGD/R + Exo + miR-124- + Rap group was noticeably reduced; however, GLT-1 protein expression was markedly improved ($p < 0.001$) (Fig. 5D–F). The findings showed that MSC-Exos' inhibitory effect on pS6 expression and its promoting effect on GLT-1 expression was reversed by miR-124 inhibitor in astrocytes after OGD/R, whereas the aforementioned conditions were reversed once again by rapamycin.

4. Discussion

Rodent studies have proved that MSC-Exos are effective treatment for ischemic stroke, which could attenuate cerebral infarction area, reduce neurological damage, and rescue synaptic communication, neuronal plasticity, spatial memory, and learning [19,20]. A recent clinical trial also confirmed the safety and feasibility of intraparenchymal injection of MSC-Exos in the treatment of ischemic stroke [21]. Nevertheless, the therapeutic mechanisms of MSC-Exo in the treatment of ischemic stroke are far from clear, which limits its clinical application and development.

Based on preclinical studies, MSC-Exos could promote the repair of nerve tissue damage by improving nerve axon regeneration, angiogenesis, anti-inflammatory and immune regulation after ischemic stroke [22,23]. However, whether MSC-Exos play a therapeutic role by inhibiting excitotoxic injury has not been reported. Excitotoxic injury is a crucial mechanism for nerve cell destruction after ischemic stroke, in which GLT-1 plays a critical role. Recent studies have confirmed that ceftriaxone, hormones, and mild hypothermia could effectively improve neurological damage by regulating GLT-1 expression after is-

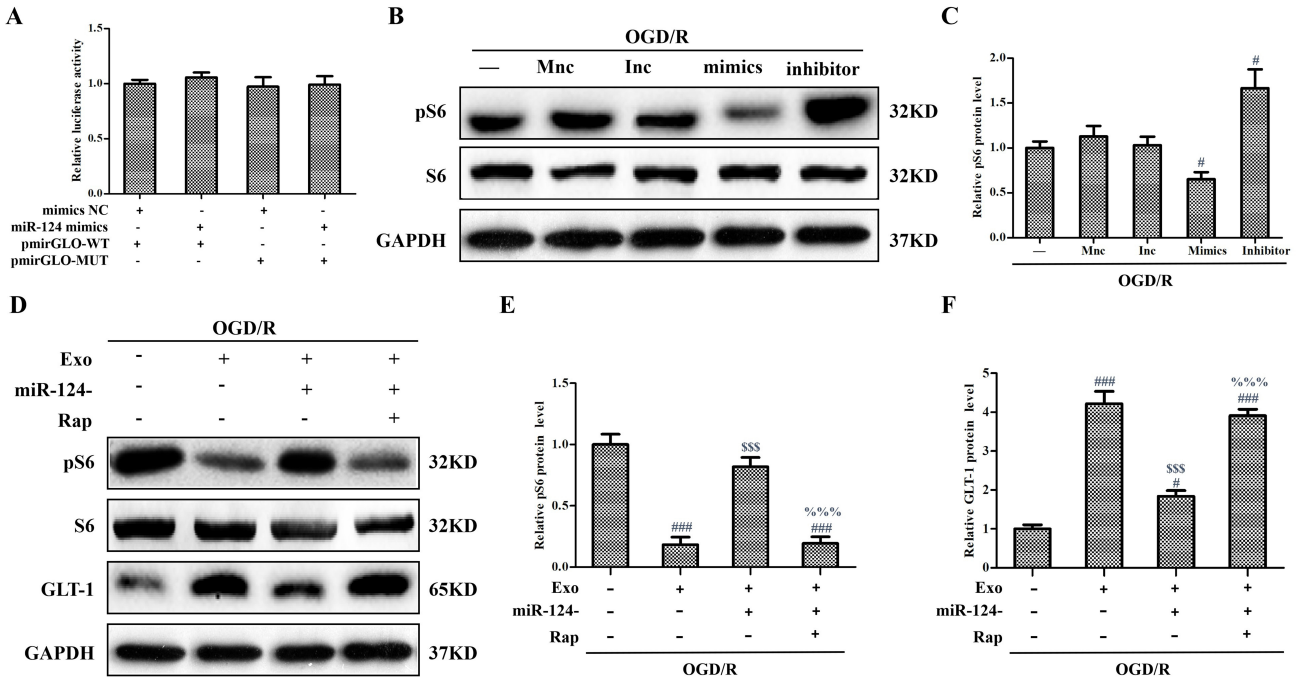

Fig. 5. MSC-Exos upregulated GLT-1 expression via the miR-124/mTOR pathway in astrocytes after OGD/R. (A) The luciferase reporter assay showed an interaction between GLT-1 and miR-124. The luciferase activity of reporter containing the wild-type or mutant 3′UTR could not be inhibited by miR-124 mimics. (B,C) The effects of miR-124 mimics (miR-124+) and inhibitor (miR-124-) on the mTOR pathway (pS6 and S6) protein expression in astrocytes after OGD/R. (D–F) The pS6 and GLT-1 protein expressions intervened by MSC-Exos and/or miR-124 inhibitor and/or rapamycin were detected by WB. Compared with the OGD/R group, # $p < 0.05$, ### $p < 0.001$; compared with the OGD/R + Exo group, $$$ $p < 0.001$; compared with the OGD/R + Exo + miR-124- group, %%% $p < 0.001$. As a ratio to the internal, the relative protein expression was displayed (n = 3).

chemic stroke [24,25]. In this investigation, GLT-1 mRNA expression gradually increased with increased MSC-Exos concentration. Additionally, MSC-Exos at a concentration of 100 μg/mL effectively inhibited the decrease of GLT-1 protein expression after OGD/R, which demonstrates that MSC-Exos upregulates GLT-1 expression in astrocytes after OGD/R.

MSC-Exos have been found to contribute to neuroprotection in ischemic stroke by regulating miRNA, which alleviates inflammation and oxidative stress, enhances axon-myelin remodeling, and inhibit microglial M1 polarization against ischemic stroke by affecting miRNAs expressions such as miR-15a-5p, miR-17-92 and miR-223-3p [26–28]. Moreover, there are no reports about whether MSC-Exos affects GLT-1 expression by regulating miRNA following ischemic stroke. It has been reported that GLT-1 expression is promoted by downregulating miR-107 expression after OGD/R [29]. Previous research by the authors has confirmed that GLT-1 expression can be regulated by miR-124 in astrocytes after OGD/R [17]. This study shows that with increased MSC-Exos concentration, GLT-1 and miR-124 expression were correlated in astrocytes after OGD/R. The upregulation of GLT-1 induced by MSC-Exos was suppressed by miR-124 inhibitor after OGD/R, which shows that MSC-Exos affects GLT-1 expression via regulating miR-124 in astrocytes after OGD/R. Nevertheless, a luciferase reporter assay revealed that GLT-1 and miR-124 did not directly regulate one another.

The mTOR pathway activated with increasing expression of phosphorylated protein pS6 regulates neuronal autophagy, angiogenesis, axonal growth, and neuronal activity after ischemic stroke [30]. This study found that the pS6 protein was highly expressed after OGD/R. MSC-Exos accelerates burn wound healing, resists oxidative stress, and improve synaptic plasticity by regulating the mTOR pathway [31,32]. In this study, it was investigated whether MSC-Exos significantly inhibited the upregulation of pS6 after OGD/R, which showed that MSC-Exos mediated the mTOR pathway after OGD/R. Meanwhile, the mTOR pathway inhibitor, rapamycin, also notably decreased pS6 expression with significantly higher GLT-1 expression in astrocytes after OGD/R. These findings indirectly inferred that MSC-Exos upregulated GLT-1 expression via the mTOR pathway in astrocytes injured by OGD/R.

The mTOR pathway is mediated by a variety of upstream regulators, among which miRNA is important. It has been reported that the mTOR pathway participates in the role of miR-34a on improving brain aging and atrophy induced by high glucose [33]. A recent study has shown

that miR-124-3p improves the biological behavior of males diagnosed with major depression by regulating the mTOR pathway [16]. This study confirms that the mTOR pathway is regulated by miR-124 in astrocytes after OGD/R. Further findings demonstrate that the miR-124 inhibitor reverses MSC-Exos' inhibiting effect on pS6 expression and promoting effect on GLT-1 expression in astrocytes after OGD/R. Meanwhile, the above conditions could be reversed again by the mTOR pathway inhibitor, rapamycin, which shows that the mTOR pathway, as a target of miR-124, is involved in the regulation of MSC-Exos on GLT-1 expression in astrocytes after OGD/R.

5. Conclusions

This study reported here show that miR-124 and the mTOR pathway are involved in the regulation of MSC-Exos on GLT-1 expression in astrocytes after OGD/R. miR-124 does not directly target GLT-1. Rather, MSC-Exos upregulates GLT-1 expression via the miR-124/mTOR pathway in astrocytes injured by OGD/R.

However, there are some limitations in this study. For example, whether miR-124-modified MSC-Exos have better therapeutic effects needs to be further verified. Meanwhile, the relevant mechanisms need to be further demonstrated in animal experiments.

Availability of Data and Materials

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

Author Contributions

WH and YF participated in the conceptualization, methodology and software. WH, YF and CJ performed the investigation. JJ, WJ, and HH were involved in data analysis. WH was involved in writing original draft. WH and YF were involved in writing, reviewing, and editing. JS was involved in conceptualized and designed the study. JS was involved in funding acquisition and collected resources. JS performed supervision. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Wistar rats were bought from the Animal Experiment Center of the Affiliated Wuxi People's Hospital of Nanjing Medical University (Jiangsu, China). The animal experimentation was approved by the Ethics Committee of Wuxi People's Hospital (approval number: XJS22001). Animal suffering was kept to a minimum during all procedures since sodium pentobarbital anesthesia was employed.

Acknowledgment

Not applicable.

Funding

This research was supported by the National Natural Science Foundation of China (81701216), Wuxi Taihu Lake Talent Plan, Supports for Leading Talents in Medical and Health Profession (2020THRC-DJ-SNW), Reserve Talents of Double Hundred Talent Plan (HB2020021), General Program of Wuxi Medical Center, Nanjing Medical University (WMCG202320), General Program of Wuxi Commission of Health (M202225).

Conflict of Interest

The authors declare no conflict of interest.

References

[1] Suda S, Nito C, Yokobori S, Sakamoto Y, Nakajima M, Sowa K, *et al*. Recent Advances in Cell-Based Therapies for Ischemic Stroke. International Journal of Molecular Sciences. 2020; 21: 6718.

[2] Levy ML, Crawford JR, Dib N, Verkh L, Tankovich N, Cramer SC. Phase I/II Study of Safety and Preliminary Efficacy of Intravenous Allogeneic Mesenchymal Stem Cells in Chronic Stroke. Stroke. 2019; 50: 2835–2841.

[3] Nikfarjam S, Rezaie J, Zolbanin NM, Jafari R. Mesenchymal stem cell derived-exosomes: a modern approach in translational medicine. Journal of Translational Medicine. 2020; 18: 449.

[4] Hade MD, Suire CN, Suo Z. Mesenchymal Stem Cell-Derived Exosomes: Applications in Regenerative Medicine. Cells. 2021; 10: 1959.

[5] Dumbrava DA, Surugiu R, Börger V, Ruscu M, Tertel T, Giebel B, *et al*. Mesenchymal stromal cell-derived small extracellular vesicles promote neurological recovery and brain remodeling after distal middle cerebral artery occlusion in aged rats. GeroScience. 2022; 44: 293–310.

[6] Gregorius J, Wang C, Stambouli O, Hussner T, Qi Y, Tertel T, *et al*. Small extracellular vesicles obtained from hypoxic mesenchymal stromal cells have unique characteristics that promote cerebral angiogenesis, brain remodeling and neurological recovery after focal cerebral ischemia in mice. Basic Research in Cardiology. 2021; 116: 40.

[7] Falcucci RM, Wertz R, Green JL, Meucci O, Salvino J, Fontana ACK. Novel Positive Allosteric Modulators of Glutamate Transport Have Neuroprotective Properties in an *in Vitro* Excitotoxic Model. ACS Chemical Neuroscience. 2019; 10: 3437–3453.

[8] Chao N, Li ST. Synaptic and extrasynaptic glutamate signaling in ischemic stroke. Current Medicinal Chemistry. 2014; 21: 2043–2064.

[9] Pajarillo E, Rizor A, Lee J, Aschner M, Lee E. The role of astrocytic glutamate transporters GLT-1 and GLAST in neurological disorders: Potential targets for neurotherapeutics. Neuropharmacology. 2019; 161: 107559.

[10] Nematipour S, Vahidinia Z, Nejati M, Naderian H, Beyer C, Azami Tameh A. Estrogen and progesterone attenuate glutamate neurotoxicity via regulation of EAAT3 and GLT-1 in a rat model of ischemic stroke. Iranian Journal of Basic Medical Sciences. 2020; 23: 1346–1352.

[11] Lai RC, Yeo RWY, Lim SK. Mesenchymal stem cell exosomes. Seminars in Cell & Developmental Biology. 2015; 40: 82–88.

[12] Bang OY, Kim EH, Cho YH, Oh MJ, Chung JW, Chang WH, *et al*. Circulating Extracellular Vesicles in Stroke Patients Treated

with Mesenchymal Stem Cells: A Biomarker Analysis of a Randomized Trial. Stroke. 2022; 53: 2276–2286.
[13] Morel L, Regan M, Higashimori H, Ng SK, Esau C, Vidensky S, *et al*. Neuronal exosomal miRNA-dependent translational regulation of astroglial glutamate transporter GLT1. The Journal of Biological Chemistry. 2013; 288: 7105–7116.
[14] Villa-González M, Martín-López G, Pérez-Álvarez MJ. Dysregulation of mTOR Signaling after Brain Ischemia. International Journal of Molecular Sciences. 2022; 23: 2814.
[15] Han X, Yang L, Du H, Sun Q, Wang X, Cong L, *et al*. Insulin Attenuates Beta-Amyloid-Associated Insulin/Akt/EAAT Signaling Perturbations in Human Astrocytes. Cellular and Molecular Neurobiology. 2016; 36: 851–864.
[16] Wang Q, Zhao G, Yang Z, Liu X, Xie P. Downregulation of microRNA 124 3p suppresses the mTOR signaling pathway by targeting DDIT4 in males with major depressive disorder. International Journal of Molecular Medicine. 2018; 41: 493–500.
[17] Huang WY, Jiang C, Ye HB, Jiao JT, Cheng C, Huang J, *et al*. miR-124 upregulates astrocytic glutamate transporter-1 via the Akt and mTOR signaling pathway post ischemic stroke. Brain Research Bulletin. 2019; 149: 231–239.
[18] Huang W, Lv B, Zeng H, Shi D, Liu Y, Chen F, *et al*. Paracrine Factors Secreted by MSCs Promote Astrocyte Survival Associated with GFAP Downregulation After Ischemic Stroke via p38 MAPK and JNK. Journal of Cellular Physiology. 2015; 230: 2461–2475.
[19] Zhao J, Deng H, Xun C, Chen C, Hu Z, Ge L, *et al*. Therapeutic potential of stem cell extracellular vesicles for ischemic stroke in preclinical rodent models: a meta-analysis. Stem Cell Research & Therapy. 2023; 14: 62.
[20] Deng M, Xiao H, Zhang H, Peng H, Yuan H, Xu Y, *et al*. Mesenchymal Stem Cell-Derived Extracellular Vesicles Ameliorates Hippocampal Synaptic Impairment after Transient Global Ischemia. Frontiers in Cellular Neuroscience. 2017; 11: 205.
[21] Dehghani L, Khojasteh A, Soleimani M, Oraee-Yazdani S, Keshel SH, Saadatnia M, *et al*. Safety of Intraparenchymal Injection of Allogenic Placenta Mesenchymal Stem Cells Derived Exosome in Patients Undergoing Decompressive Craniectomy Following Malignant Middle Cerebral Artery Infarct, A Pilot Randomized Clinical Trial. International Journal of Preventive Medicine. 2022; 13: 7.
[22] Farzaneh M, Khoshnam SE. Functional roles of mesenchymal stem cell-derived exosomes in ischemic stroke treatment. Current Stem Cell Research & Therapy. 2022. (online ahead of print)
[23] Rezaie J, Feghhi M, Etemadi T. A review on exosomes application in clinical trials: perspective, questions, and challenges. Cell Communication and Signaling: CCS. 2022; 20: 145.
[24] Malik AR, Willnow TE. Excitatory Amino Acid Transporters in Physiology and Disorders of the Central Nervous System. International Journal of Molecular Sciences. 2019; 20: 5671.
[25] Gao Z, Zhang Z, Bian Q, Li Y, Ma D, Liu Z, *et al*. Mild hypothermia protects rat cortical neurons against oxygen-glucose deprivation/reoxygenation injury via the PI3K/Akt pathway. Neuroreport. 2021; 32: 312–320.
[26] Yang H, Chen J. Bone marrow mesenchymal stem cell-derived exosomes carrying long noncoding RNA ZFAS1 alleviate oxidative stress and inflammation in ischemic stroke by inhibiting microRNA-15a-5p. Metabolic Brain Disease. 2022; 37: 2545–2557.
[27] Xin H, Liu Z, Buller B, Li Y, Golembieski W, Gan X, *et al*. MiR-17-92 enriched exosomes derived from multipotent mesenchymal stromal cells enhance axon-myelin remodeling and motor electrophysiological recovery after stroke. Journal of Cerebral Blood Flow and Metabolism: Official Journal of the International Society of Cerebral Blood Flow and Metabolism. 2021; 41: 1131–1144.
[28] Zhao Y, Gan Y, Xu G, Hua K, Liu D. Exosomes from MSCs overexpressing microRNA-223-3p attenuate cerebral ischemia through inhibiting microglial M1 polarization mediated inflammation. Life Sciences. 2020; 260: 118403.
[29] Yang ZB, Luo XJ, Ren KD, Peng JJ, Tan B, Liu B, *et al*. Beneficial effect of magnesium lithospermate B on cerebral ischemia-reperfusion injury in rats involves the regulation of miR-107/glutamate transporter 1 pathway. European Journal of Pharmacology. 2015; 766: 91–98.
[30] Hadley G, Beard DJ, Couch Y, Neuhaus AA, Adriaanse BA, DeLuca GC, *et al*. Rapamycin in ischemic stroke: Old drug, new tricks? Journal of Cerebral Blood Flow and Metabolism: Official Journal of the International Society of Cerebral Blood Flow and Metabolism. 2019; 39: 20–35.
[31] Ramhormozi P, Mohajer Ansari J, Simorgh S, Nobakht M. Bone Marrow-Derived Mesenchymal Stem Cells Combined with Simvastatin Accelerates Burn Wound Healing by Activation of the Akt/mTOR Pathway. Journal of Burn Care & Research: Official Publication of the American Burn Association. 2020; 41: 1069–1078.
[32] Zhang Y, Chopp M, Liu XS, Katakowski M, Wang X, Tian X, *et al*. Exosomes Derived from Mesenchymal Stromal Cells Promote Axonal Growth of Cortical Neurons. Molecular Neurobiology. 2017; 54: 2659–2673.
[33] Chen P, Chen F, Lei J, Li Q, Zhou B. Activation of the miR-34a-Mediated SIRT1/mTOR Signaling Pathway by Urolithin A Attenuates D-Galactose-Induced Brain Aging in Mice. Neurotherapeutics: the Journal of the American Society for Experimental NeuroTherapeutics. 2019; 16: 1269–1282.