The delivery of miR-21a-5p by extracellular vesicles induces microglial polarization via the STAT3 pathway following hypoxia-ischemia in neonatal mice

Abstract
Extracellular vesicles (EVs) from mesenchymal stromal cells (MSCs) have previously been shown to protect against brain injury caused by hypoxia-ischemia (HI). The neuroprotective effects have been found to relate to the anti-inflammatory effects of EVs. However, the underlying mechanisms have not previously been determined. In this study, we induced oxygen-glucose deprivation in BV-2 cells (a microglia cell line), which mimics HI in vitro, and found that treatment with MSCs-EVs increased the cell viability. The treatment was also found to reduce the expression of pro-inflammatory cytokines, induce the polarization of microglia towards the M2 phenotype, and suppress the phosphorylation of selective signal transducer and activator of transcription 3 (STAT3) in the microglia. These results were also obtained in vivo using neonatal mice with induced HI. We investigated the potential role of miR-21a-5p in mediating these effects, as it is the most highly expressed miRNA in MSCs-EVs and interacts with the STAT3 pathway. We found that treatment with MSCs-EVs increased the levels of miR-21a-5p in BV-2 cells, which had been lowered following oxygen-glucose deprivation. When the level of miR-21a-5p in the MSCs-EVs was reduced, the effects on microglial polarization and STAT3 phosphorylation were reduced, for both the in vitro and in vivo HI models. These results indicate that MSCs-EVs attenuate HI brain injury in neonatal mice by shuttling miR-21a-5p, which induces microglial M2 polarization by targeting STAT3.

Key Words: extracellular vesicles; hypoxia-ischemia; mesenchymal stromal cells; microglia; miR-21a-5p; neuroinflammation; oxygen-glucose deprivation; STAT3

Introduction
Brain injury due to neonatal hypoxia-ischemia (HI) is a major cause of death and long-term neurodevelopmental disability (Ziemka-Nalecz et al., 2017). Following HI, there is evidence that proinflammatory mediators are released, which lead to neuroinflammation; this can alter neuronal function and contributes to permanent HI-induced brain damage (Liu and McCullough, 2013; Hagberg et al., 2015). Microglia are the main effectors of neuroinflammation following HI. They can be polarized into proinflammatory M1 or anti-inflammatory M2 states (Colton, 2009), and the balance between these two phenotypes plays an important role in regulating the neuroinflammation, as well as in maintaining brain homeostasis.

Mesenchymal stromal cells (MSCs) possess broad immunoregulatory properties and have potential for treating diseases that are associated with inflammation (Bernardo and Fibbe, 2013; Teixeira and Salgado, 2020; Han et al., 2021). For example, a recent study showed that human umbilical cord-derived MSCs exert anti-diabetic effects and alleviate islet dysfunction in a mouse model of type 2 diabetes, by inducing a switch from the M1 to the M2 macrophage phenotype (Yin et al., 2018). It is thought that the immunomodulatory properties of MSCs relate to paracrine signaling (Bazzoni et al., 2020). In support of this, it has been found that MSCs stimulated by a lipopolysaccharide (LPS) secrete certain factors that affect macrophage function (Crisostomo et al., 2008; Bernardo and Fibbe, 2013). Recently, extracellular vesicles (EVs) have been recognized as an important paracrine factor produced by MSCs, which contribute to the beneficial effects of MSCs (Bazzoni et al., 2020). The EVs can be classified as small (50–100 nm), medium (100 nm–1 μm), or large (1–5 μm) (Thery et al., 2018). It has been suggested that MSCs-derived EVs promote an immunosuppressive response by promoting the polarization of macrophages from the M1 to the M2 phenotype, regulating the immature dendritic cells, and secreting anti-inflammatory cytokines (Bazzoni et al., 2020). Recent research suggests that MSCs-EVs can be used to reduce ischemic brain damage. MSCs-EVs from human Wharton’s jelly were found to protect neuronal cells against oxygen-glucose deprivation (OGD)-induced apoptosis (Joerger-Messerli et al., 2018). MSCs-EVs were also found to suppress LPS-induced inflammation mediated by BV-2 cells (Thomi et al., 2019).

Studies have shown that EVs contain microRNAs (miRNAs), which are taken up by recipient cells and can affect the cell fate (Xin et al., 2012, 2022). The delivery of miR-21a-5p by extracellular vesicles induces microglial polarization via the STAT3 pathway following hypoxia-ischemia in neonatal mice. Neural Regen Res 17(10):2238-2246.

2238 | NEURAL REGENERATION RESEARCH | Vol 17 | No. 10 | October 2022
Tissue collection and preparation for immunohistochemistry and immunofluorescence assays

Three days after the HI, the mice (n = 4 per group) were killed, and the brains were quickly removed and paraffin-embedded. The paraffin-embedded sections were cut into 4 μm-thick coronal paraffin sections, for the regions −1.6 mm to −2.0 mm from the bregma, using a paraffin microtome (Thermo Fisher Scientific, Waltham, MA, USA). These were then stained for immunofluorescence and immunohistochemistry assays.

The immunofluorescence assays were carried out as described in a previous study (McCullough et al., 2005). For this, the brain tissue sections were dewaxed and antigen repaired, and the slides were incubated with the following primary antibodies (Table 1) overnight at 4°C: rabbit monoclonal anti-pSTAT3 antibody (phosphorylated transcription factor, 1:100), mouse anti-ib-1 (1:100), rabbit anti-CD16 (1:100), rabbit anti-CD4 (1:100), rabbit anti-CD11b (1:100), rabbit anti-CD11c (1:100), rabbit anti-CD26 (1:100), and rabbit anti-CD45 (1:100). The slides were then incubated with secondary antibodies (Table 2) for 30 minutes at 4°C: Alexa Fluor® 488 AffiniPure goat anti-mouse IgG (H+L; 1:100), Alexa Fluor® 594 AffiniPure goat anti-rabbit IgG (H+L; 1:100), Alexa Fluor® 647 and Alexa Fluor® 488 AffiniPure goat antimouse IgG (H+L; 1:100). Finally, DAPI staining was carried out at room temperature for 5 minutes to reveal the cell nuclei (Beyotime Institute of Biotechnology, Jiangsu, China). Fluorescence images were obtained using Pannoramic MIDI (3D HISTECH, Budapest, Hungary).

The immunohistochemical assays were carried out as described in a previous study (Chu et al., 2019). For this, the brain slices were incubated overnight at 4°C with mouse anti-ib-1 (1:100) primary antibody (Table 1). The slices were then treated with enzyme-labeled goat anti-mouse/rabbit IgG at room temperature for 30 minutes. The antibody binding was analyzed using a DAB kit (Gene Tech, Shanghai, China), and the slides were observed using Pannoramic MIDI. The number of endpoints per ib-1 (microglial) cell and the length of the cell processes in the infarct core (n = 4 per group) were determined for three fields (1000x) using a Fiji software (National Institutes of Health, Bethesda, MD, USA; Schindelin et al., 2012).

Culture and identification of MSCs

Bone marrow MSCs were harvested from 30 C57BL/6j male mice (4 weeks old), as previously described and modified (Wang et al., 2009). To summarize, the mice were euthanized by an intraperitoneal injection of a 2% sodium pentobarbital solution in a volume of 20 ml/kg, and the femurs and tibia were carefully dissected. The bone marrow was flushed with 10 ml of FBS (Thermo Fisher Scientific) to remove the adherent tissue. The FBS was centrifuged at 100,000 × g for 6 hours. The supernatant was removed, and the pellet was resuspended in an appropriate amount of FBS-free FBS (Beckman Coulter, Brea, CA, USA) and used for the analyses.

Ten million cells were seeded onto a plastic dish, and the culture medium was replaced every 3 days. After 3 weeks, the cells were observed under a light microscope and analyzed for their morphology.

Flow cytometry analysis

Flow cytometry analysis (FACS) was used to detect surface markers on the MSCs (Soleimani and Nadri, 2009). The MSCs were incubated with the following fluorescein isothiocyanate (FITC)-conjugated antibodies (Thermo Fisher Scientific, China) and stained with calcein AM (50 μM) for 1 hour at 37°C. The cell density was adjusted to 10^6 cells/ml, and 1 μl/ml fluorescein diacetate (FD) was added to the suspension. After 5 minutes at 37°C, the cells were washed twice with PBS containing 1% FBS and analyzed by flow cytometry. The live cells were gated by their forward and side scatter properties.

Acquisition of EVs-free FBS

FBS (Thermo Fisher Scientific) was centrifuged at 100,000 × g for 6 hours. The resulting supernatant was used as EVs-free FBS.

Harvesting and identification of MSCs-EVs, and analysis of contents

A total EVs isolation kit (iEV, #1001622, 20xScience, Christchurch, New Zealand) was used to isolate and identify the EVs, as previously described (Kim et al., 2020). To summarize, when the MSCs density reached approximately 60%, the complete medium (Thermo Fisher Scientific) was replaced with the EVs-free FBS medium (DMEM/F-12 containing 10% EVs-free FBS, 2 mM L-glutamine, 100 μM penicillin, and 100 μg/ml streptomycin) for 24 hours. The cell supernatant was then collected and centrifuged at 5752 × g for 30 minutes. The EVs were pelleted from the supernatant at 100,000 × g for 6 hours.
Research Article

Biolegend inhibitor Servicebio (Wuhan, China)

Table 1 | Primary antibodies used in the study

| Antibody | Dilution | RRID | Cat# | Supplier |
|----------|----------|------|------|----------|
| Mouse monoclonal β-actin antibody | 1:1000 | – | TA-09 | Zhongshan Golden Bridge Biotechnology (Beijing, China) |
| Mouse monoclonal Iba1 antibody | 1:1000 | – | GB12105 | Servicebio (Wuhan, China) |
| Rabbit polyclonal IL-1β antibody | 1:1000 | – | sc-7884 | Santa Cruz Biotechnology (Santa Cruz, CA, USA) |
| Rabbit polyclonal Arginase-1 antibody | 1:1000 | – | 9819S | Cell Signaling Technology, Danvers, MA, USA |
| Rabbit monoclonal p-STAT3 antibody | 1:1000 | – | 10253-2-AP | Proteintech Group (Rosemont, IL, USA) |
| Rabbit polyclonal STAT3 antibody | 1:1000 | – | ab125011 | Abcam (Cambridge, UK) |
| Rabbit polyclonal TSG101 antibody | 1:1000 | – | 10427-2-AP | Proteintech Group |
| Rabbit polyclonal Calnexin antibody | 1:1000 | – | 9145T | Cell Signaling Technology |
| Rabbit monoclonal CD9 antibody | 1:1000 | – | ab97276 | Abcam |
| Mouse monoclonal GM130 antibody | 1:1000 | – | sc-55590 | Santa Cruz Biotechnology |
| Mouse monoclonal Lamin A/C antibody | 1:1000 | – | sc-376484 | Santa Cruz Biotechnology |
| Mouse monoclonal Cyclin C1 antibody | 1:1000 | – | sc-514435 | Santa Cruz Biotechnology |
| FITC anti-mouse/rat CD29 antibody | 1:200 | AB-312882 | 102205 | Biogend (San Diego, CA, USA) |
| PE/Cy7 anti-mouse human CD44 antibody | 1:200 | AB-830786 | 103029 | Biogend |
| FITC anti-mouse CD31 antibody | 1:200 | AB-312900 | 102405 | Biogend |
| APC anti-mouse Sca-1 antibody | 1:200 | AB-10639725 | 108125 | Biogend |
| FITC anti-mouse/human CD11b antibody | 1:200 | AB-312788 | 101206 | Biogend |
| APC anti-mouse CD45 antibody | 1:200 | AB-312977 | 103112 | Biogend |
| FITC anti-mouse CD14 antibody | 1:200 | AB-940578 | 123307 | Biogend |
| PE anti-mouse CD19 antibody | 1:200 | AB-313642 | 115507 | Biogend |
| PerC anti-mouse Ly6G antibody | 1:200 | AB-2616998 | 127653 | Biogend |
| APC anti-mouse CD117 antibody | 1:200 | AB-313220 | 105811 | Biogend |

Table 2 | Secondary antibodies used in the study

| Antibody | Dilution | RRID | Cat# | Supplier |
|----------|----------|------|------|----------|
| Alexa Fluor® 594 AffiniPure goat Anti-rabbit IgG (H+L) | 1:100 | AB_2338059 | 111-585-003 | Jackson ImmunoResearch Inc (PA, USA) |
| Alexa Fluor® 488 AffiniPure goat Anti-mouse IgG (H+L) | 1:100 | AB_2338840 | 115-545-003 | Jackson ImmunoResearch Inc (PA, USA) |
| HRP-labeled goat anti-rabbit IgG (H+L) | 1:10000 | None | ZB-2301 | Zhongshan Golden Bridge Biotechnology |
| HRP-labeled goat anti-mouse IgG (H+L) | 1:10000 | None | ZB-2305 | Zhongshan Golden Bridge Biotechnology |

HRP: Horse radish peroxidase.

Jiangsu Province, China). BV-2 cells were transfected with the pmirGLO-WT/Mut-STAT3, miR-21a-5p mimics, and miR-21a-5p NC (GenePharma), using Lipofectamine 2000 according to the manufacturer’s instructions (Thermo Fisher Scientific). A Dual-Luciferase Reporter Assay System (Promega Corporation, Madison, WI, USA) was used to detect firefly and renilla luciferase activity.

RNA isolation and quantitative reverse transcription-polymerase chain reaction

The total RNA in the BV-2 cells and brain tissue was extracted using an Ultrapore RNA Kit (Cat# 01761/20114-1, CWBio, Beijing, China) according to

A western blot analysis was used to detect the common markers of EVs, such as CD9 and tumour susceptibility gene 101 (TSG-101; Thery et al., 2018), and the morphology of the EVs was observed using a transmission electron microscope (TEM, Hitachi, Tokyo, Japan). The qNano platform (Izon Sciences Ltd., Christchurch, New Zealand) was used to determine the size and concentration of the EVs.

Table 2 | Secondary antibodies used in the study

| Antibody | Dilution | RRID | Cat# | Supplier |
|----------|----------|------|------|----------|
| Alexa Fluor® 594 AffiniPure goat Anti-rabbit IgG (H+L) | 1:100 | AB_2338059 | 111-585-003 | Jackson ImmunoResearch Inc (PA, USA) |
| Alexa Fluor® 488 AffiniPure goat Anti-mouse IgG (H+L) | 1:100 | AB_2338840 | 115-545-003 | Jackson ImmunoResearch Inc (PA, USA) |
| HRP-labeled goat anti-rabbit IgG (H+L) | 1:10000 | None | ZB-2301 | Zhongshan Golden Bridge Biotechnology |
| HRP-labeled goat anti-mouse IgG (H+L) | 1:10000 | None | ZB-2305 | Zhongshan Golden Bridge Biotechnology |
the manufacturer’s instructions. MSCs-EVs were isolated using ExoQuick-TCTM (System Biosciences), and the RNA was extracted using a SeraMiv EVs RNA Extraction Kit (System Biosciences, Mountain View, CA, USA). Complementary DNA (cDNA) was synthesized using a ReverTra Ace qRT-PCR and cDNA Synthesis Kit (Cat# FSQ-101, TOYOBO, Osaka, Japan). The quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using SYBR Green PCR master mix (Cat# PC3301, Aidlab Biotechnologies, Beijing, China) in a Bio-Rad IQ5 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) with gene-specific primer pairs (GenePharma, Shanghai, China). The RNA isolation and quantitative reverse transcription polymerase chain reaction (qRT-PCR) was carried out as follows: 2 minutes at 95°C for denaturation, followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 60°C. The expression level was standardized relative to the internal level (β-actin or U6), and the relative expression level was calculated using the 2^(-ΔΔCt) method (Xin et al., 2020). The sequences used in the present study are shown in Table 3.

Table 3 | Primers used in polymerase chain reaction

| Gene    | Primer sequence (5’-3’) | product size (bp) |
|---------|------------------------|-------------------|
| β-actin | F: CAA GCA CCT TGG AAG AGG | 118            |
| R: GAA GCC AAA CAC AGG ATC | 27 |
| iNOS    | F: CAA GCA CCT TGG AAG AGG | 118            |
| R: CTT TAC AGT CCT GGA CCC | 18 |
| CD206   | F: CAA GAG ATT GGC TTA GGT | 110            |
| R: CTG CGT GGG CAA AGA CTG | 18 |
| IL-10   | F: CCA AGG ATT AAT GCC AGA G | 110            |
| R: TTT TCA CAG GGA AGA AAT CGG | 18 |
| TGF-β   | F: TGC GTC TGT AGA AGT GAT | 107            |
| R: CGG CAA GGA AGA GCC ACT CA | 22 |
| TNF-a   | F: CGG GGT CCT CTA TGG AGG | 118            |
| R: ACC CTG AGC CAT AAT CCC CT | 27 |
| IL-1β   | F: TGC CAA TTT TGC AGT GAT | 107            |
| R: TGA TGG CTT GGG AGA ATG | 22 |
| U6      | F: CAG ACA TTA ATT TCC AGG ACG | 118            |
| R: ACG AAT TGG GTC GTC ACC | 27 |
| mmu-miR-21-5p | Sense: AGG TGG TGT AGG TGT ACG TGT | 21 |
| Antisense: AAT GGT TGT TCT CCA TCT T | 21 |
| mics negative control | Sense: UUC UCA CGA CCG GUC ATT G | 21 |
| Antisense: ACA GGA CAC GGU CGG ATT G | 21 |
| mmu-miR-21-5p mimics | Sense: UAG CUC UUC AUC ACG AUG UGA A | 21 |
| Antisense: AAC ACC ACG GUG AAG AGU U | 21 |
| mmu-miR-21-5p inhibitor | UCA ACA UCA GUC UGA UCA GGU | 21 |
| Inhibitor negative control | UCA ACA CCA UUG UCA CAC UCC | 21 |

Reverse transcription polymerase chain reaction analysis
RNA was extracted from the MSCs-EVs and cDNA was synthesized in reverse transcription polymerase chain reaction (RT-PCR) as described above. The cDNA for miR-21-5p was then amplified using PCR with specific primers (Table 3). The PCR reaction products were separated using electrophoresis on 1.2% agarose/TAE gel (Biowest, Loire Valley, France) containing 0.1% GoldView (v/v), run at 90 V for 30 minutes. The resulting image was visualized with the Tanon Imaging System (Tanon-2500, Tanon Science & Technology Co., Shanghai, China).

Western blot analysis
MSCs-EVs, BV-2 cells, or the right cortex of the HI group were homogenized for 10 minutes in RIPA buffer (Cat# P00138, Beyotime Institute of Biotechnology, Jiangsu, China) containing protease/phosphatase inhibitors and PMSF (Cat# ST506-2, Beyotime). The homogenate was then centrifuged at 13,800 × g for 10 minutes at 4°C. The proteins were separated using SDS-PAGE gels, run at 100 V for 30 minutes and at 120 V for 1 hour. They were then transferred to a PVDF membrane (Cat# IPVH00010, Millipore, Billerica, MA, USA) for at least 1 hour at 300 mA (note that the specific transfer time depends on the target molecular weight). The PVDF membrane was then blocked with 5% non-fat milk for 1 hour. The blots were probed with the following primary antibodies (Table 2) overnight at 4°C: rabbit polyclonal STAT3 (transcription factor) antibody, rabbit monoclonal p-STAT3 (phosphorylated transcription factor) antibody, rabbit polyclonal interleukin-1β (IL-1β, proinflammatory factor) antibody, rabbit polyclonal arginase-1 (inflammatory factor) antibody, rabbit monoclonal TSG101 antibody (a marker for EVs), rabbit monoclonal CD9 antibody, rabbit monoclonal TSG101 antibody (a marker for EVs), mouse monoclonal GM130 antibody (mitochondrial marker), mouse monoclonal cytochrome C1 antibody (cytosome C1), mouse monoclonal lamin A/C antibody (nuclear marker), and mouse monoclonal β-actin antibody (internal control). The membranes were incubated at room temperature for 1 hour with the secondary antibodies (Table 2) and then detected using a Tanon Imaging System (Tanon-4600).
miR-21a-5p in MSCs-EVs attenuates the inflammatory response following OGD in BV-2 cells

EVs-mediated miRNA transport has been proposed to be important for immunomodulatory properties. In this study, to investigate the miR-21a-5p in the MSCs-EVs (D1), the miR-21a-5p levels were found to be significantly lower. This was found for 1-hour OGD ($t = 2.687, df = 10, P < 0.05$), 3-hour OGD ($t = 2.969, df = 10, P < 0.05$), and 5-hour OGD ($t = 1.655, df = 10, P > 0.05$). Treatment with MSCs-EVs was found to substantially increase the level of miR-21a-5p (OGD for 3 hours; $F_{(2,15)} = 20.783, P < 0.001$; post hoc $P < 0.001$; Figure 4C).

To determine the role of miR-21a-5p in the neuroprotection conferred by MSCs-EVs, MSCs-EVs were pretreated with a miR-21a-5p inhibitor (EVs-miR-21a-5p inhibitor) or its negative control (EVs-miR-21a-5p). The qRT-PCR showed that the miR-21a-5p inhibitor substantially decreased the expression of miR-21a-5p in the MSCs-EVs ($F_{(2,15)} = 162.026, P < 0.001$; Figure 4D). The miR-21a-5p inhibitor was also found to reverse the increase in miR-21a-5p levels seen following MSCs-EVs treatment post-OGD ($F_{(2,15)} = 15.773, P < 0.001$; post hoc $P < 0.01$; Figure 4E).

The cell viability was also assessed. It was found that the EVs-mir-21a-5p inhibitor significantly reduced the BV-2 cell viability following OGD, compared with the EVs-miR-21a-5p ($F_{(2,20)} = 31.199, P < 0.001$; post hoc $P < 0.001$; Figure 4F). The EVs-miR-21a-5p inhibitor was also found to suppress the anti-inflammatory effects of EVs-miR-21a-5p, as shown by the mRNA levels of TNF-$\alpha$ ($F_{(2,20)} = 37.149, P < 0.001$; post hoc $P < 0.001$), IL-1β ($F_{(2,20)} = 23.528, P < 0.001$; post hoc $P < 0.001$), iNOS ($F_{(2,20)} = 8.357, P < 0.01$; post hoc $P < 0.001$; IL-10 ($F_{(2,20)} = 8.387, P < 0.01$; post hoc $P < 0.01$), and CD206 ($F_{(2,20)} = 12.117, P < 0.001$; post hoc $P < 0.05$; Figure 4G).

Figure 1 | Identification of EVs and MSCs.
(A) Transmission electron microscopy image of EVs (red arrows). Scale bar: 200 nm. (B) Nanoparticle tracking analysis of MSCs-EVs using the qNano platform. (C) Western blot analysis of TSG101, CD9, GM130, Lamin A/C, Cytochrome C1, and Calnexin in the MSCs and EVs. (D) Immunofluorescence analysis showing the internalization of PKH67-EVs by the BV-2 cells (green arrow) that had undergone 3-hour OGD. Scale bar: 20 µm. (D1) Magnification of the boxed region in D showing the location of the PKH67 hotspots. Scale bar: 5 µm. (E) Fluorescence microscopy image of the MSCs showing a typical spindle-like morphology. Scale bar: 20 µm. (F) Images showing the MSCs’ capacity to differentiate into adipocytes and osteoblasts. Scale bars: 100 µm. (G) Flow cytometry analysis of MSCs surface markers (positive markers: CD44, CD29, and Sca-1; negative markers: CD11b, CD45, CD31, CD117, ly66, CD19, CD14, and CD4). EV: Extracellular vesicle; MSC: mesenchymal stromal cell; TSG101: tumour susceptibility gene 101.

Figure 2 | EVs promote M2 polarization in BV-2 cells after OGD.
(A) qRT-PCR analysis of pro- and anti-inflammatory cytokine mRNA expression in BV-2 cells after 1-, 3-, or 5-hour OGD followed by 24-hour reoxygenation. $n = 6$ per group. (B) qRT-PCR analysis of the mRNA levels of IL-1β, iNOS, TNFα, IL-10, TGF-β, and CD206 in the presence or absence of MSCs-EVs. Cells had undergone 3-hour OGD followed by 24-hour reoxygenation. Cells incubated under standard cell culture conditions formed the “control” group. Graphs show the mean ± SD. All of the experiments were carried out on six separate samples. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ (independent samples t-test) in A and B; ***$P < 0.001$ (one-way analysis of variance with Bonferroni correction) in C. EV: Extracellular vesicle; MSC: mesenchymal stromal cell; OGD: oxygen-glucose deprivation.
MiR-21a-5p in MSCs-EVs targets the STAT3 signaling pathway following OGD in BV-2 cells

We ran database searches to identify potential targets of miR-21a-5p that may be associated with microglial polarization and inflammation. We chose to focus on the miR-21a-5p/STAT3 pathway, as this has been shown to be critical for modulating the immune system and inflammation (Satriotomo et al., 2006; Fang and Zhang, 2020). We found that STAT3 phosphorylation (p-STAT3) increased following 1-hour OGD (t = −4.209, df = 6, P < 0.01), 3 h OGD (t = −3.198, df = 6, P < 0.05), and 5 h OGD (t = −4.348, df = 6, P < 0.01; Figure 5A). Treatment with MSCs-EVs significantly decreased the levels of p-STAT3 (F1,12,3 = 8.368, P < 0.001; post hoc p < 0.05; Figure 5A); however, this decrease was no longer apparent when a miR-21a-5p inhibitor was added to the MSCs-EVs (post hoc p < 0.05; Figure 5C).

A TargetScan analysis predicted that the miR-21a-5p would bind to the 3′-UTR of STAT3 (Figure 5D). We found that miR-21a-5p mimics decreased the luciferase activity associated with WT STAT3 3′ UTR, but not the luciferase activity associated with MUT STAT3 3′ UTR, in BV-2 cells (t = 25.649, df = 10, P < 0.001; Figure 5E).

MSCS-EVs inhibit microglial activation

Immunohistochemical staining showed that the Iba-1+ cells in the sham group had a ramified shape. In the HI group, the microglia were activated and had a rounded, amoeboid-like appearance. The administration of MSCS-EVs significantly suppressed the microglial activation. The number of endpoints per Iba-1+ cell was found to be significantly lower in the HI group compared with the sham group (F2,18 = 14.672, P < 0.01; post hoc P < 0.01). It was also found that the length of the Iba-1+ cell processes was shorter in the HI group than in the sham group (F2,18 = 20.744, P < 0.001; post hoc P < 0.001). This difference was no longer apparent following treatment with MSCS-EVs (Figure 6).

MSCS-EVs suppress HI-induced neuro-inflammation and promot M2 microglial polarization

Treatment with MSCS-EVs was found to attenuate the mRNA levels of pro-inflammatory cytokines 72 hours after HI, including IL-1β (F1,12,3 = 143.562, P < 0.001; post hoc P < 0.001) and TNF-α (F1,12,3 = 119.605, P < 0.001; post hoc P < 0.001; Figure 7A); the treatment also increased the mRNA levels of anti-inflammatory cytokines, including CD206 (F1,12,3 = 32.918, P < 0.001; post hoc P < 0.001) and TGF-β (F1,12,3 = 122.783, P < 0.001; post hoc P < 0.001; Figure 7A). Analysis of the protein levels revealed that the treatment significantly decreased the protein levels of IL-1β (F1,12,3 = 9.967, P < 0.01, post hoc P < 0.05) and increased the protein levels of arginase-1 (F1,12,3 = 7.362, P < 0.05; post hoc P < 0.05; Figure 7B).

Microglial polarization was assessed using immunofluorescence staining 3 days after the HI. It was found that the number of M1 phenotypes (Iba1+CD16+ cells) in the right cortex was significantly lower in the HI + EVs group compared with the HI group (F2,16 = 54.827, P < 0.001; post hoc P < 0.05; Figure 7C), whereas the number of M2 phenotypes (Iba1+CD206+ cells) was significantly higher (F2,16 = 21.274, P < 0.001; post hoc P < 0.05; Figure 7C).

Figure 4 | miR-21a-5p in MSCS-EVs attenuates the inflammatory response after OGD in BV-2 cells.

(A) RT-PCR analysis of the levels of miR-21a-5p in EVs, EVs pretreated with RNase A, EVs pretreated with Triton X-100, EVs pretreated with Triton X-100 and RNase A, and RT-negative samples. (B) qRT-PCR analysis of the levels of miR-21a-5p in EVs after 1-, 3-, 5-, and 24-hour OGD followed by 24-hour reoxygenation. The control group was cultured normally without OGD. (C) RT-PCR analysis of the levels of miR-21a-5p in the presence or absence of MSCs-EVs. The cells had undergone 3-hour OGD followed by 24-hour reoxygenation. (D) qRT-PCR analysis of the levels of miR-21a-5p in the different EVs (EVs, EVs-miR-21a−, and EVs-miR-21a−/−). (E) qRT-PCR analysis of the levels of miR-21a-5p following treatment with EVs, EVs-miR-21a−, or EVs-miR-21a−/−. The cells had undergone 3-hour OGD followed by 24-hour reoxygenation. (F) The viability of BV-2 cells, assessed using CCK8, following treatment with EVs, EVs-miR-21a−, or EVs-miR-21a−/−. The cells had undergone 3-hour OGD followed by 24-hour reoxygenation. (G) qRT-PCR analysis of the mRNA levels of TNF-α, IL-1β, iNOS, CD206, IL-10, and TGF-β for cells treated with no EVs, EVs, EVs-miR-21a−, or EVs-miR-21a−/−. The cells had undergone 3-hour OGD followed by 24-hour reoxygenation. Graphs show the mean ± SD. The experiments were carried out on six separate samples. *P < 0.05 (independent samples t-test) in B; **P < 0.01, ***P < 0.001 (one-way analysis of variance with Bonferroni correction) in C–G. EV: Extracellular vesicle; IL: interleukin; iNOS: inducible nitric oxide synthase; MSC: mesenchymal stromal cell; OGD: oxygen-glucose deprivation; qRT-PCR: quantitative reverse transcription-polymerase chain reaction; RT-PCR: reverse transcription PCR; TGF-β: transforming growth factor-β; TNF-α: tumor necrosis factor-α.

Figure 5 | MiR-21a-5p in MSCS-EVs targets STAT3 signaling pathway after OGD in BV-2 cells.

(A) Western blot analysis of phosphorylated STAT3 (p-STAT3) and STAT3 in BV-2 cells after 1-, 3-, and 5-hour OGD followed by 24-hour reoxygenation. The control cells were cultured normally without OGD. (B) Western blot analysis of the levels of p-STAT3 and STAT3 in the presence or absence of MSCS-EVs. The BV-2 cells had undergone 3-hour OGD followed by 24-hour reoxygenation. (C) Western blot analysis of the levels of p-STAT3 and STAT3 in BV-2 cells treated with EVs, EVs-miR-21a−/−, or EVs-miR-21a−/−. The cells had undergone 3-hour OGD followed by 24-hour reoxygenation. (D) The sequence of mouse miR-21a-5p and its predicted binding site within the STAT3 3′ untranslated region (3′-UTR); the mutated sequence is also shown. (E) Luciferase activity in BV-2 cells transfected with luciferase plasmids containing STAT3 3′-UTR or MUT STAT3 3′-UTR and treated with miR-21a-5p mimics or miR-21a-5p NC. The cells were lysed to measure the relative luciferase activity. The experiments were carried out on four independent samples. Graphs show the mean ± SD; *P < 0.05; **P < 0.01; ***P < 0.001 (independent samples t-test) in A and E; *P < 0.05 (one-way analysis of variance with Bonferroni correction) in B and C. EV: Extracellular vesicle; MSC: mesenchymal stromal cell; OGD: oxygen-glucose deprivation.
MIR-21a-5p in MSCs-EVs mediates the neuroprotective effects through the STAT3 signaling pathway

To show that the MSCs-EVs' anti-inflammatory effects are mediated by MIR-21a-5p, mice were injected with either PBS, MSCs-EVs, EVs-MIR-21a<sub>incub</sub> or EVs-MIR-21a<sub>incub</sub>. As previously found (Chu et al., 2020; Xin et al., 2020), the MSCs-EVs significantly decreased the brain water content (F<sub>2,8</sub> = 50.806, P < 0.001; post hoc P < 0.001) and infarct volumes (F<sub>2,8</sub> = 189.879, P < 0.001; post hoc P < 0.001) in the HI neonatal mice (Additional Figure 1). In contrast, this was not found for the EVs-MIR-21a<sub>incub</sub> (Additional Figure 2).

As in a previous study (Satomi et al., 2006), p-STAT3 staining was found to be localized in the Iba<sub>1</sub> microglia/macrophages in the hemisphere ipsilateral to the injury (Additional Figure 3). In line with our in vitro results, the EVs treatment significantly reduced p-STAT3 (F<sub>2,9</sub> = 17.488, P < 0.01; post hoc P < 0.01) in the right cortex 3 days following the HI (Figure 8A). This effect was significantly smaller with the EVs-MIR-21a<sub>incub</sub> (F<sub>2,11</sub> = 32.603, P < 0.001; post hoc P < 0.01; Figure 8B).

The qRT-PCR showed that the treatment with EVs-MIR-21a<sub>incub</sub> upregulated the mRNA levels of pro-inflammatory cytokines, including IL-1β (F<sub>2,10</sub> = 10.615, P < 0.001; post hoc P < 0.01) and TNFα (F<sub>2,11</sub> = 14.258, P < 0.001; post hoc P < 0.001), while the mRNA levels were downregulated for CD206 (F<sub>2,9</sub> = 68.897, P < 0.001; post hoc P < 0.001) and TGF-β (F<sub>2,10</sub> = 23.655, P < 0.001; post hoc P < 0.001; Figure 8C). It was also found that the EVs-MIR-21a<sub>incub</sub> treatment increased the protein level of IL-1β (F<sub>2,9</sub> = 9.264, P < 0.01; post hoc P < 0.05) and reduced the protein level of Arginase-1 (F<sub>2,11</sub> = 33.357, P < 0.001; post hoc P < 0.001) compared with the EVs-MIR-21a<sub>incub</sub> treatment group (Figure 8D).

Figure 6 | MSCs-EVs decrease microglial activation in the cortex ipsilateral to the HI.
Upper panels: Immunohistochemistry images of microglia with Iba-1 staining in the sham, HI, and HI+EVs groups. Green arrowheads show the resting microglia (ramified microglia) and red arrowheads show the activated microglia (amoeboid shape). Scale bar: 25 µm. Lower panels: Quantification of the number of endpoints per Iba-1 cell and the length of the cell processes in the infarct core. All of the experiments were carried out on four independent samples. Graphs show the mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001 (one-way analysis of variance with Bonferroni correction). EV: Extracellular vesicle; HI: hypoxia-ischemia; IL: interleukin; MSC: mesenchymal stromal cell.

Figure 7 | MSCs-EVs promote M2 microglial polarization and suppress HI-induced neuroinflammation in the cortex ipsilateral to the HI.
(A) Quantitative reverse transcription polymerase chain reaction analysis of the mRNA levels of IL-1β, TNFα, TGF-β, and CD206 in the presence or absence of MSCs-EVs, 72 hours post-HI. Results are shown for the cortex ipsilateral to the HI. (B) Western blot analysis of the levels of IL-1β and Arginase-1 in the presence or absence of MSCs-EVs, 72 hours post-HI. Results are shown for the cortex ipsilateral to the HI. (C) Representative photographs showing immunofluorescence staining of Iba-1 (green), CD16 (red), and CD206 (red) in the cortex ipsilateral to the HI, 72 hours post-HI. Scale bars: 50 µm. Graphs show the mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001 (one-way analysis of variance with Bonferroni correction). All of the experiments were carried out on six (A) or four (B, C) independent samples. Double cell means Iba-1'CD16' cells or Iba-1'CD206' cells. EV: Extracellular vesicle; HI: hypoxia-ischemia; IL: interleukin; MSC: mesenchymal stromal cell; TGF-β: transforming growth factor-β; TNF-α: tumor necrosis factor α.

Figure 8 | MIR-21a-5p in MSCs-EVs provides neuroprotection through STAT3 signaling pathway in the cortex ipsilateral to the HI.
(A) Western blot analysis of the levels of STAT3 and p-STAT3 in the presence or absence of MSCs-EVs, 72 hours post-HI. Results are shown for the cortex ipsilateral to the HI. (B) Western blot analysis of the levels of p-STAT3 and STAT3 in the presence or absence of EVs, EVs-MIR-21a<sub>incub</sub>, or EVs-MIR-21a<sub>incub</sub>. Results are shown for the cortex ipsilateral to the HI. (C) Quantitative reverse transcription polymerase chain reaction analysis of the mRNA levels of IL-1β, TNF-α, TGF-β, and CD206 in the presence or absence of EVs, EVs-MIR-21a<sub>incub</sub>, or EVs-MIR-21a<sub>incub</sub>. Results are shown for the cortex ipsilateral to the HI. Graphs show the mean ± SD; *P < 0.05, **P < 0.01, ***P < 0.001 (one-way analysis of variance with Bonferroni correction). All of the experiments were carried out on four (A, B, D) or six (C) independent samples. EV: Extracellular vesicle; HI: hypoxia-ischemia; IL: interleukin; MSC: mesenchymal stromal cell; p-STAT3: phosphorylated STAT3; STAT3: signal transducer and activator of transcription 3; TGF-β: transforming growth factor-β; TNF-α: tumor necrosis factor α.
Discussion

MSCs-EVs have recently been recognized for their potential to treat injuries involving brain and tissue damage and ischemia and stroke. In this study, we found that MSCs-EVs alleviated the neuroinflammation following HI, and that this was associated with the polarization of microglia from a pro-inflammatory to an anti-inflammatory state, as seen both in vivo and in vitro. We obtained evidence that miR-21a-5p in the MSCs-EVs may change the microglial polarization, which would then regulate neuroinflammation and maintain brain homeostasis. STAT3 may thus represent a new potential therapeutic target for the treatment of HI.

MSCs-EVs suppress neuroinflammation by changing microglia from a pro-inflammatory to an anti-inflammatory state

The therapeutic effects of MSCs-EVs relate to their immunosuppressive properties. In a previous study, microvesicles from MSCs were found to reduce LPS-induced inflammatory responses in BV-2 cells (Liu et al., 2017). Another study used an animal model of Alzheimer’s disease and found that MSCs-EVs suppressed the activation of microglia, polarized the microglia towards an anti-inflammatory phenotype, and increased the dendritic spine density of neurons (Louroso et al., 2020). MSCs-EVs have also been found to significantly reduce microglial activation and prevent reactive astrogliosis following LPS-stimulated brain injury (Drommelschmidt et al., 2017).

In our previous work, we found that MSCs-EVs administered by intracardiac injection following HI were localized in the microglia (Chu et al., 2020; Xin et al., 2021). This treatment was found to decrease neuroinflammation, suppressing the expression of osteopontin in the microglia/macrophages (Xin et al., 2021). In the present study, we found that the MSCs-EVs were internalized by BV-2 cells following OGD, and resulted in a decrease in BV-2 cell apoptosis, a reduction in the expression of M1 microglial markers (iNOS and TNF-a), and an increase in the expression of M2 microglial markers (including CD206, TGF-β, and IL-10). These results were confirmed in our in vivo study, where MSCs-EVs treatment was found to significantly decrease the mRNA levels of IL-1β and TNF-a, and increase the mRNA levels of CD206 and TGF-β. In addition, the EVs treatment was found to decrease the protein levels of IL-1β and increase the protein levels of arginase-1. The number of microglia with the M1 phenotype (Iba1-CD16 cells) was also found to be lower following the MSCs-EVs treatment, while the number of microglia with the M2 phenotype (Iba1-CD206 cells) was found to be higher in the cortex ipsilateral to the ligation. These results indicate that MSCs-EVs modulate the microglial polarization and could potentially be used as a therapeutic treatment for HI brain damage.

Anti-inflammatory mechanisms involving the miR-21a-5p in MSCs-EVs

One of the main mechanisms underlying the therapeutic effects of MSCs-EVs relates to the transfer of miRNA between cells. We previously reported that miR-21a-5p was highly abundant in MSCs-EVs (Xin et al., 2020), and several studies have shown that miR-21 plays an important role in the anti-inflammatory response in many diseases, such as tumors, infections, and other diseases that are associated with inflammation (Sheedy et al., 2010; Sheedy, 2015). It has been found that the overexpression of miR-21 suppresses the production of inflammatory cytokines and can also improve cardiac function following myocardial infarction (Yang et al., 2018). In an animal model of Alzheimer’s disease, EVs from hypoxia-preconditioned MSCs were found to improve learning and memory; this was attributed to improvements in synaptic function and the regulation of inflammatory responses via miR-21 (Cui et al., 2018). In another study, the overexpression of miR-21 in EVs was found to reduce cell apoptosis and improve cardiac function following myocardial infarction (Song et al., 2019).

The present study showed that treatment with MSCs-EVs markedly increased the expression of miR-21a-5p in BV-2 cells following OGD. The beneficial effect of MSCs-EVs on microglial polarization, inflammatory cytokines, and cell survival were no longer apparent when a miR-21a-5p inhibitor was administered in vitro (Thomi et al., 2019). Treatment with MSCs-EVs has also been found to suppress early inflammatory responses following a traumatic brain injury in rats by modulating the microglia/macrophage polarization (Ni et al., 2019). Following HI brain injury in newborn rats, umbilical cord MSCs-EVs have been shown to be internalized by microglia and act to reduce the neuroinflammation (Thomi et al., 2019). Treatment with MSCs-EVs has also been found to significantly reduce microgliosis and prevent reactive astrogliosis following LPS-stimulated brain injury (Drommelschmidt et al., 2017).

Limitations

Our study has some limitations. Firstly, we only examined the effects of the EVs’ miR-21a-5p on short-term HI outcomes. It remains to be determined whether long-term effects can be identified. Secondly, we did not consider whether the EVs’ miR-21a-5p affects the peripheral immune cells, and whether these may participate in the regulation of neuroinflammation. Thirdly, we could not examine whether the same therapeutic effect can be achieved using a STAT3 inhibitor (such as STAT3 siRNA); if this were found to be the case, the cost of treatment would be further reduced. Further research is needed to examine these three points.

Conclusion

The study showed that treatment with MSCs-EVs attenuated HI brain injury in neonatal mice. This was due to the transfer of miR-21a-5p, which induced the polarization of microglia to the M2 phenotype by targeting STAT3.

Acknowledgments: We thank the Animal Medicine Center of Shandong University for providing experimental animals and the Basic Medical Sciences of Shandong University for providing experimental places and instrument.

Author contributions: ZW, ZMK, YL, and ZLG made substantial contributions to data interpretation, writing and revising of the manuscript, and final revision of the manuscript. DKL conceived experiments, analyzed the data and final revision of the manuscript. DXQ made substantial contributions to laboratory work, analyzed the data, and wrote the manuscript. YZ performed Western blotting. TTL, HK, and CGG performed cell culture. XFG and WQK revised the manuscript. All authors read and approved the final manuscript.

Conflicts of interest: The authors have no financial or personal conflict of interest to disclose.

Author statement: This paper has been posted as a preprint on Research Square with doi: https://doi.org/10.21203/rs.3.rs.313905/v1, which is available from: https://assets.researchsquare.com/files/rs-313905/v1/77ee6119-c7d2-45df-8475-4ca3ac36bd6c.pdf?c=1631879647.

Availability of data and materials: All data generated or analyzed during this study are included in this published article and its supplementary information files.

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Open peer reviewer: Gokul Krishnak, The University of Arizona College of Medicine – Phoenix, USA.

Additional files: Additional Figure 2: MSCs-EVs suppress HI-induced edema and infarct in mice at 3 days post-HI insult. Additional Figure 3: p-STAT3 is localized in the Iba1+ cells microglia/macrophages in the ipsilateral hemisphere of mice.

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MSCs-EVs treatment was found to suppress the STAT3 activation, which is consistent with the polarization of microglia from a pro-inflammatory to an anti-inflammatory state following HI. We were able to show that miR-21a-5p binds directly to the STAT3 gene, using a Dual-Luciferase Reporter Assay, and we found that its expression increased in miR-21a-5p levels in microglia was associated with a decrease in activated STAT3. A miR-21a-5p inhibitor was found to counteract this effect, both in vivo and in vitro. Taken together, these results suggest that MSCs-EVs regulate microglial activation by transferring miR-21a-5p to the microglia and targeting the STAT3 pathway.

The study showed that treatment with MSCs-EVs attenuated HI brain injury in neonatal mice. This was due to the transfer of miR-21a-5p, which induced the polarization of microglia to the M2 phenotype by targeting STAT3.
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Santiago-Gil R, Zhao J; C-Editor: Zhao M; S-Editor: Li CH; L-Editor: Li CH, Song LP; T-Editor: Jia Y

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Additional Figure 1 MSCs-EVs suppresses HI-induced edema and infarct in mice at 3 days post-HI insult.

(A) Representative brain pictures. (B) Brain water content of mice. (C) Complete sets of brain slices from a representative animal (TTC staining). White indicates infarction. \( n = 4 \)/group. \( "n" \) represents the number of biological repetitions in each group. Values represent the mean ± SD. ***\( p < 0.001 \) (one-way analysis of variance with Bonferroni correction). EV: Extracellular vesicle; HI: hypoxia-ischemia; MSC: mesenchymal stromal cell.
Additional Figure 2 MiR-21a-5p regulates the neuroprotective effects of MSCs-EVs following HI.

(A) Representative brain pictures at 72 hours following HI in mice treated with vehicle (Veh) or EVs injection, including MSCs-EVs, MSCs-EVs pretreated with the miR-21a-5p inhibitor (EVs-miR-21a\textsuperscript{inhibitor}), or the miR-21a-5p inhibitor negative control (EVs-miR-21a\textsuperscript{INC}). (B) Brain water content of mice. (C) Complete sets of brain slices from a representative animal (TTC staining). White indicates infarction. $n = 4$/group. “n” represents the number of biological repetitions in each group. Values represent the mean $\pm$ SD. **$P < 0.01$, ***$P < 0.001$ (one-way analysis of variance with Bonferroni corrections). EV: Extracellular vesicle; HI: hypoxia-ischemia; MSC: mesenchymal stromal cell.
Additional Figure 3  p-STAT3 is localized in the Iba1+ cells microglia/macrophages in the ipsilateral hemisphere of mice.

Representative photographs of immunofluorescent staining of p-STAT3 (red), Iba-1 (green) within the ipsilateral cortex at 72 hours post-HI insult. Scale bars: 50 µm. n = 4/group. “n” represents the number of biological repetitions in each group. Values represent the mean ± SD. ***P < 0.001 (independent samples t-test).

HI: Hypoxia-ischemia; p-STAT3: phosphorylated signal transducer and activator of transcription 3.