Exosomal miR-23b from bone marrow mesenchymal stem cells alleviates oxidative stress and pyroptosis after intracerebral hemorrhage

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Abstract
Our previous studies showed that miR-23b was downregulated in patients with intracerebral hemorrhage (ICH). This indicates that miR-23b may be closely related to the patho-physiological mechanism of ICH, but this hypothesis lacks direct evidence. In this study, we established rat models of ICH by injecting collagenase VII into the right basal ganglia and treating them with an injection of bone marrow mesenchymal stem cell (BMSC)-derived exosomal miR-23b via the tail vein. We found that edema in the rat brain was markedly reduced and rat behaviors were improved after BMSC exosomal miR-23b injection compared with those in the ICH groups. Additionally, exosomal miR-23b was transported to the microglia/macrophages, thereby reducing oxidative stress and pyroptosis after ICH. We also used hemin to mimic ICH conditions in vitro. We found that phosphatase and tensin homolog deleted on chromosome 10 (PTEN) was the downstream target gene of miR-23b, and exosomal miR-23b exhibited antioxidant effects by regulating the PTEN/Nrf2 pathway. Moreover, miR-23b reduced NLRP3 inflammasome-mediated pyroptosis, thereby promoting neurologic function recovery in rats with ICH.

Key Words: bone marrow mesenchymal stem cells, exosomal miRNAs; intracerebral hemorrhage; miR-23b; neuroinflammation; NLRP3 inflammasome; Nrf2; oxidative stress; PTEN; pyroptosis.

Introduction
Intracerebral hemorrhage (ICH) is a cerebrovascular disease that presents with high morbidity and mortality, and it accounts for 15% of strokes globally (Xi et al., 2006; Deng et al., 2021; Zhu et al., 2022). Brain injury caused by the hematoma mass mechanical effects, which can cause neurologic injury, and ICH secondary brain injury can cause further serious and persistent neurological disorders. The mechanisms of ICH secondary brain injury are complicated and include oxidative stress, neuroinflammation, excitotoxicity, and cytotoxicity. These mechanisms are connected to each other and collectively lead to brain edema and brain injury (Zhu et al., 2019). Pyroptosis, a process of programmed inflammatory death, has crucial effects in ICH-induced neuroinflammation through activating the nucleotide-binding oligomerization domain-like receptor family pyrin domain containing 3 (NLRP3)-caspase-1 inflammasomes or other inflammasomes (Fang et al., 2020). Studies demonstrated that ICH-induced NLRP3 inflammasomes could be activated by robust reactive oxygen species (ROS) overproduction, and the ROS scavenger alleviated the NLRP3 inflammatory response through reducing oxidative stress, which indirectly protected neuronal cell from death and further reduced ICH injury (Tang et al., 2020; Chen et al., 2022). Thus, effective therapeutic strategies to alleviate pyroptosis and oxidative damage, therefore to protect neuronal cells from death are urgently needed.

Exosomes are important in bone marrow mesenchymal stem cell (BMSC) communication, and they exhibit protective roles in various diseases including ICH, and the researchers showed that exosomes effectively promoted neurological recovery after ICH by alleviating neuroinflammation and inhibiting neuronal apoptosis (Duan et al., 2020; Shi et al., 2021). Exosomes can to convey information by transferring microRNAs (miRNAs), long non-coding RNAs, and proteins, and thus, attention should be paid to miRNA effects and function, long non-coding RNAs, and proteins in exosomes (Zhang et al., 2015). miRNAs are small non-coding RNA molecules that exert their biological function in various diseases by reducing target gene expression through translational inhibition or mRNA destabilization (Bushart and Cohen, 2007). We showed down-regulated miR-23b expression in patients with ICH in a previous study, which suggested the possible biological functions of miR-23b in ICH (Zhu et al., 2015; Wang et al., 2016). Besides their involvement in different cancers (Lei et al., 2021; Yang et al., 2021), miR-23b was recently found to play various anti-inflammatory roles in multiple neurological diseases (Zhang et al., 2018; Hu et al., 2019). Moreover, miR-23b in exosomes were shown to be derived from BMSCs, which can prevent intracranial aneurysm formation by maintaining the Th17/Treg immune balance. However, evidence regarding the implication of BMSC-exosomal miR-23b in ICH is lacking. Therefore, this study aimed to explore the functions of BMSC-exosomal miR-23b in ICH-induced oxidative stress and pyroptosis and to investigate the molecular mechanisms using animal and cell models.

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In Vivo Experiments (ARRIVE) guidelines (Percie du Sert et al., 2016) were followed. Protocols were approved by the Laboratory Animal Ethics Committee at China Medical University (No. 20170088) on March 8, 2017. All experiments were designed and reported in accordance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines (Percie du Sert et al., 2016) for animal experimentation. A previous study (Roquer et al, 2016) showed that men than women had ICH (52.4% vs. 47.6%). Because of the higher incidence of ICH in men compared with that in women, we chose male rats for our study. One hundred and forty-four male Wistar rats (SPF level, 250–280 g, 8–12 weeks old) were obtained from Chang Sheng Biotechnology Co. Ltd. (Bexin, Liaoning, China; license No. SCXK (Liaoy) 2019-0001). All rats were housed under standard conditions (temperature 25 ± 1°C; humidity, 50 ± 5%), and a regular 12-hour light/dark cycle was used, and they had free access to food and water. There were three rats in each cage. The study procedure is shown in Figure 1.

Figure 1  Animal experimental flow chart. Eto 0.05%: exosomes derived from BMSCs transfected with miR-23b; Eto0.05%: exosomes derived from BMSCs transfected with miR-NC; GSH: reduced glutathione; GSSG: oxidized glutathione disulfide; HE: hematoxin-eosin; ICH: intracerebral hemorrhage; MDA: malondialdehyde; PBS: phosphate-buffered saline; ROS: reactive oxygen species; TUNEL: terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling.

Methods

Animals

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Behavioral assessment and brain edema evaluation

To evaluate neurological behavior in rats including the sensory and motor function, corner tests and forelimb placement tests were performed on days 1, 3, and 7 after the injection. The rats were administered with exosomes for 5 days before the behavioral test, rats were approached in a 30° corner and had to turn to the left or right to exit the corner. We recorded the choice of turning side for ten trials per rat. Forelimb placement tests were performed as described in the previous study (Xu et al., 2018). Each rat was tested for ten trials. The percentage of trials in which the rat placed the appropriate forelimb on the edge of the counterpont in response to vibrissa stimulation was calculated. Rats were anesthetized using 50 mg/kg 2% sodium pentobarbital (Cat# P5761, Sigma) via intraperitoneal injection, and then they were sacrificed by decapitation. Ipsilateral hemisphere tissue was dissected on day 3 and weighed immediately on an electronic balance (Sartorius, Gottingen, Niedersachsen, Germany) to determine the brain wet weight. The tissue was then heated at 100°C for 1 day to determine the dry weight. The water content (%) was obtained as (wet weight – dry weight) / wet weight × 100.

Hematoxylin-eosin staining

Rats were sacrificed at day 3 for the histology study and perfused with normal saline and 4% paraformaldehyde. Brain samples were dissected, soaked with graded sucrose, and frozen sequentially by a slicer. A hematoxylin-eosin (H&E) staining (Cat# 11203-3, Scienbio, Beijing, China) was used to stain brain coronal slides (8 μm). Briefly, the slides were stained with hematoxylin for 4 minutes and washed with distilled water. Next, the slides were incubated with the differentiation fluid for 20 seconds and washed with water for 30 minutes. Finally, the slides were stained with eosin for 30 seconds and sealed with neutral balsam.

Terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling and immunofluorescence staining

The terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling (TUNEL) assay was used to identify apoptotic cells from perihematomal brain region using in situ death detection kits to detect TUNEL-positive cells, as previously described (Hu et al., 2019) (No. 11684795910, Roche, Grenzach Wyhlem, Germany). Briefly, after fixation, the slides were permeabilized for 5 minutes and washed with PBS three times. The slides were then incubated in 50 μl of TUNEL reaction solution for 1 hour in a dark chamber. Next, slides were washed with PBS and counterstained with 4,6-diamidino-2-phenylindole (DAPI). For immunofluorescence staining, the frozen brain slides were rewarmed at 37°C and then incubated with 0.3% Triton X-100 and blocked. Rabbit anti-ionized calcium binding adapter molecule 1 (Iba1; 1:200, Abcam, Waltham, MA, USA, Cat# ab178846, RRID: AB_2636859) was applied as the primary antibody and incubated with the sections at 4°C overnight. Goat anti-rabbit IgG(H+L)-Alexa Fluor® 594 (1:1000, Thermo Fisher Scientific, Cat# A-11008, RRID: AB_1413700) was incubated with DAPI for 1 hour at 37°C. Next, we selected three slides per rat and counted the numbers of positive cells in five random fields per slice under the fluorescence microscope (Olympus, Tokyo, Japan).

Reactive oxygen species staining

Frozen sections were stained using the GENMED kit to evaluate the ROS in the perihematomal brain region (GMS100161.2, GENMED, Arlington, MA, USA; Guo et al., 2021). For ROS staining, slides were washed with cleaning reagent A and incubated with working reagent for 20 minutes at 37°C. To detect ROS in cells stimulated by hemin conditions, we diluted DCFH-DA (500 μM) in PBS at 10 μM to acquire the working concentration. The cells were incubated with working reagent at 37°C for 20 minutes and washed with DME. ROS staining was visualized using a fluorescence microscope.

Measurement of MDA activity, SOD activity, and GSH/GSSG content

The malondialdehyde (MDA), superoxide dismutase (SOD), and reduced glutathione/oxidized glutathione disulfide (GSH/GSSG) levels in perihematomal brain tissue were evaluated using the following kits: malondialdehyde activity kit (S0139, Beyotime), superoxide dismutase activity kit (S0088, Beyotime), and reduced glutathione oxidized glutathione disulfide kit (S0053, Beyotime), respectively, in accordance with the manufacturer’s instructions.

Pro-inflammatory cytokines and cell viability detection

Cytokines, including interleukin-1β (IL-1β) and interleukin-18 (IL-18) in perihematomal brain tissues and cell supernatant (collected from cultured BV2 cells without or with treatment) were measured. We performed the IL-1β and IL-18 ELISA kits (NO. R&D, Minneapolis, MN, USA) and interleukin-1 ELISA kits (NO. R&D, Minneapolis, MN, USA) using Microplate Reader (SpectraMax M5, Molecular Devices, CA, USA) and Enzyme Immunoassay Reader (Varioskan Flash).
The neuronal viability and death rates were estimated using a cell counting kit-8 (CCK8) kit (CK04, Dojing, Tokyo, Japan) and lactate dehydrogenase (LDH) assay kit (KT024448, KeyGen, Nanjing, China), as described previously (Hu et al., 2015). The results (mean±SD) of CCK8 and LDH detection were presented as standard deviation (SD). Behavioral data were analyzed using the Kruskal-Wallis test with Dunn’s multiple comparison post hoc test. The statistical significance between two groups was evaluated using the Student’s t-test. The statistical significance among multiple groups was analyzed using one-way analysis of variance (ANOVA) with the Newman-Keuls post hoc analysis. Statistical significance was set at \( P < 0.05 \). GraphPad Prism 7.0 (GraphPad software, San Diego, CA, USA) was used to analyze the data.

**Results**

**Overexpression of miR-23b in BMSCs and Identification of exosomes derived from BMSCs**

miR-23b was upregulated in the BMSC group transfected with lentivirus carrying miR-23b (LV-miR-23b) (Figure 2A). The transmission electron microscope revealed that the exosomes derived from the BMSCs exhibited a round cup-shaped and complete structure (Figure 2B). Western blot analysis further identified positive expression of the exosomal biomarkers CD63, TS101, and CD81 in the exosomes derived from the BMSCs (Figure 2C). As shown in Figure 2D, we observed a higher miR-23b level in exosomes from BMSCs transfected with miR-23b (exo-miR-23b), suggesting that the overexpressed miR-23b could be delivered to BMSC-derived exosomes.

**BMSC-exosomal miR-23b improves behavioral functions and reduces brain injuries induced by ICH**

To explore the effects of the BMSC-derived exosomes carrying miR-23b in ICH rats, the same volume of the exo-miR-23b (+), PBS, or PBS was injected, and an equal volume of miR-23b (-) was injected in the perihematomal brain tissue in the exo-miR-23b (-) group (Figure 3A). To observe the distribution of the BMSC exosomes, we labeled the exosomes with PKH67 green fluorescence and the microglia/macrophages with Iba1 staining. We found that the exosomes were delivered successfully into the microglia/macrophages in the perihematomal region (Figure 3B). The neurological deficits were evaluated using the corner tests and limb placement assessments. ICH induction increased the frequency of right turns on days 1, 3, and 7 compared with that in the sham group, and the increase was mitigated by exo-miR-23b administration on day 7 (Figure 3C). Similar neurological function improvements were observed using limb placement tests (Figure 3D). The brain water content of the ipsilateral hemisphere showed a significant increase after ICH induction, which was attenuated by exo-miR-23b or exo-miR-23b administration (Figure 3E). Brain tissue morphology in ICH rats was evaluated using H&E staining. Non-regularly arranged neuronal cells, deep staining, and a decreased number of nuclei with pyknotic nuclei infiltration were observed in the ICH group compared with those in the sham group. Additionally, the large pathological deteriorations induced by ICH were attenuated in both exo-miR-23b and exo-miR-23b groups. The fewest histological impairments were observed in rats that received exo-miR-23b administration (Figure 3F).

**BMSC-exosomal miR-23b reduces oxidative stress in the ICH rat brain**

ROS levels and oxidative stress-related proteins in the perihematomal brain tissue were increased after ICH induction compared with that in the sham group, but it was decreased by exo-miR-23b administration compared with that in the sham group (Figure 5A). Major antioxidant enzyme SOD activity in brain tissue was elevated in the exo-miR-23b group compared with that in the ICH group (Figure 5B). GSH and GSSG ratio significantly increased compared with that in the ICH group, but it was decreased by exo-miR-23b administration compared with that in the ICH group (Figure 5C). The SOD activity and GSH/GSSG ratio means that there was elevated oxidative stress. The ICH group exhibited a decreased GSH/GSSG ratio compared with that in the sham group. However, exo-miR-23b administration significantly increased the ratio compared with that in the ICH group (Figure 5D). Collectively, the results confirmed the antioxidant effect of exosomal miR-23b.

**BMSC-exosomal miR-23b attenuates pyroptosis via inhibiting NLRP3 inflammasome activation in ICH rats**

The effect of exo-miR-23b on cell pyroptosis was evaluated using RT-qPCR and Western blot. Consistent with previous studies (Hu et al., 2020; Zheng and Kanneganti, 2020), our results showed that NLRP3 inflammasome and pyroptosis were induced by ICH (Figure 6A–D). However, exo-miR-23b administration repressed the increase in NLRP3, cleaved caspase-1, and GSDMD-N compared with that in the ICH group (Figure 6A and F). Mature IL-1β and IL-18, which are NLRP3 inflammasome effectors, were further decreased in the exo-miR-23b group compared with those in the ICH group (Figure 6G and H). Collectively, the exosomal miR-23b hampered pyroptosis by inhibiting NLRP3 inflammasome activation induced by ICH.

**BMSC-exosomal miR-23b attenuates oxidative stress in vitro**

We stably expressed as BV2 cells with 60 μM hemin for 24 hours to mimic ICH conditions in vitro, and exosomes were incubated separately with hemin-stimulated microglia BV2 cells; PBS incubation was set as the control. The results showed that the miR-23b level in the exo-miR-23b group was higher than that in the other groups (Figure 7A). Oxidative stress conditions using ROS, MDA, SOD, and GSH/GSSG levels. BV2 cells treated with 60 μM hemin induced the increase in intracellular ROS, which was reversed by the treatment with the antioxidant NAC, confirming that the increase in ROS was induced by hemin (Figure 7E and F). Mature IL-1β and IL-18 were induced to further increase the ROS levels induced by hemin (Figure 7B and C). Similar results were also observed in MDA measurements (Figure 7D). SOD levels were markedly decreased by hemin stimulation, and increased significantly by the SOD levels compared with that in the groups (Figure 7E). However, exo-miR-23b administration reversed the decrease in the GSH/GSSG ratio induced by hemin was reversed by exo-miR-23b administration (Figure 7F).
BMSC-exosomal miR-23b improves behavioral functions and reduces ICH-induced brain injury.

(A) miR-23b levels in different rat groups after exosomes administration. (B) Immunofluorescence staining showed that PKH67 dye staining (green, representing exosomes) was distributed in the Iba1-stained cells (red, stained by Alexa Fluor 594, representing microglia/macrophages) in both the exo-NC and exo-miR-23b groups, indicating the successful transfer of exosomes into microglia/macrophages. Scale bar: 50 μm. (C) Behavioral functions were evaluated using the corner tests. ICH group showed a higher percentage of right turns compared with sham group, indicating severe neurological deficits after ICH induction; and the increase was mitigated by exo-miR-23b administration on day 7, indicating the improvement of neurological function after exo-miR-23b administration. (D) Behavioral functions were evaluated by forelimb placement assessments. The percentage of the trials in which the rats placed the appropriate forelimb responding to vibrissae stimulation was recorded. (E) Brain water content. (F) Representative images of H&E staining. There was no difference in which the rats placed the appropriate forelimb responding to vibrissae stimulation was recorded. (E) Brain water content. (F) Representative images of H&E staining. There was no difference

Figure 3

Figure 2

Figure 4

Figure 5

BMSC-exosomal miR-23b inhibits the amount of microglia/macrophages and neuronal apoptosis in brain tissues.

(A, B) Immunofluorescence and data analysis of microglia/macrophages with Iba1 staining (red, Alexa Fluor 594) in the perihematomal brain region. There were more immunofluorescence staining showing Iba1-stained cells (microglia/macrophages) in the ICH group than that in the sham groups, and Iba1 staining decreased in the exo-NC and exo-miR-23b groups compared with that in the ICH group. (C, D) TUNEL staining (green) and data analysis of neuronal apoptosis. There were more TUNEL-stained cells (apoptotic neuronal cells) in the ICH group compared with that in the sham group. TUNEL-stained cells decreased in the exo-NC and exo-miR-23b groups compared with that in the ICH group, and the exo-miR-23b group showed significant TUNEL staining less than that in the exo-NC group. Scale bars: 50 μm. Data are shown as the mean ± SD (n = 6). *P < 0.05, **P < 0.01, vs. sham group; *P > 0.05, **P > 0.01, vs. ICH group; #P < 0.05, vs. exo-NC group (one-way analysis of variance followed by Newman-Keuls post hoc analysis). 

BMSC-exosomal miR-23b inhibits oxidative stress in brain tissues.

(A) ROS staining in each group. ROS staining showed green fluorescence. More ROS green staining was observed in the ICH group compared with that in the sham group. The ROS green staining was decreased in the exo-miR-23b and exo-miR-23b groups compared with that in the ICH group, and the exo-miR-23b group showed less ROS staining than that in the exo-NC group. Scale bars: 50 μm. (B) Relative fluorescence intensity of ROS. ICH group: PBS injection after ICH; exo-NC group: miR-NC transfected BMSC-exosome injection after ICH; exo-miR-23b group: miR-23b transfected BMSC-exosome injection after ICH. BMSC: Bone marrow mesenchymal stem cell; DAPI: 4'6-diamidino-2-phenylindole; H&E: hematoxylin-eosin; Iba1: ionized calcium binding adapter molecule 1; ICH: intracerebral hemorrhage; ns: not significant; PBS: phosphate-buffered saline; TUNEL: terminal deoxynucleotidyl transferase mediated dUTP nick-end labeling.

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(A) miR-23b expression in BMSCs detected by RT-qPCR after lentivirus transfection. (B) Transmission electron microscope scanning images of exosomes. The exosomes derived from the BMSCs showed a round cup-shaped and complete structure. Scale bars: 100 nm. (C) Western blot bands detecting exosome markers CD63, TSG101, and CD81. (D) MiR-23b expression levels in different groups detected by RT-qPCR. Data are shown as the mean ± SD (n = 3). *P < 0.05 (Student’s t-test). BMSCs: Bone marrow mesenchymal stem cells; Exo-NC: exosomes derived from BMSCs transfected with miR-NC; RT-qPCR: reverse transcription quantitative polymerase chain reaction.

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**Figure 11**

(A) miR-23b overexpression was significantly elevated after ICH induction. (B) Pyroptosis-related protein expression was increased after ICH induction. (C, D) IL-1β (C) and IL-18 (D) levels were evaluated by ELISA after exosome administration. (E) Western blot results revealed consistent miR-23b overexpression compared with that of the miR-23b mimic group. The predicted binding between PTEN and miR-23b was observed and anti-inflammatory effects, we used Targetscan to investigate the target of miR-23b. PTEN acts as a target of miR-23b

**Figure 12**

(BMSC-exosomal miR-23b attenuates NLRP3 inflammasome-mediated pyroptosis in brain tissues.

(A) mRNA expression of pyroptosis-related genes by RT-qPCR was significantly elevated after ICH induction. (B) Pyroptosis-related protein expression after exosome administration. (G, H) IL-1β (G) and IL-18 (H) levels were assessed by ELISA after exosome administration. Data are shown as the mean ± SD (n = 6). SP < 0.05, SSP < 0.01, vs. sham group; $P < 0.05$, $PP < 0.01$, vs. ICH model group; $P < 0.05$, vs. exo*NS group. Student’s t-test in A–D; one-way ANOVA with Newman-Keuls analysis in E–H. Sham group: Control group; PBS injection after ICH; exo**NS group: miR-NC transfected BMSC-exosome injection after ICH; exo**NS group: miR-23b transfected BMSC-exosome injection after ICH. BMSC: Bone marrow mesenchymal stem cell; ELISA: enzyme-linked immunosorbent assay; GSDMD-N: N-terminal fragment of gasdermin D; ICH: intracerebral hemorrhage; IL-1β: interleukin-1β; IL-18: interleukin-1β; NLRP3: NOD-like receptor family pyrin domain containing 3; PBS: phosphate-buffered saline; ROS: reactive oxygen species; SOD: superoxide dismutase.

**BMSC-exosomal miR-23b alleviates NLRP3 inflammasome-mediated pyroptosis by regulating PTEN**

Nm23 is crucial to maintain cellular redox homeostasis, and the evidence suggested that Nrf2 activation is influenced by nuclear export and PI3K-mediated degradation (Ding et al., 2020). To explore whether PTEN/Nrf2 signaling is involved in the modulation of miR-23b in oxidative stress, we confirmed the Nrf2 nuclear translocation and downstream antioxidant gene HO-1. We found that Nrf2 nuclear translocation and the HO-1 level were increased by exo**NS administration compared with that in the hemin group (Figure 10A and B). PTEN was overexpressed by plasmid transfection. PTEN overexpression reduced Nrf2 nuclear translocation and the HO-1 level compared with that in the exo**NS group (Figure 10A and B) and inhibited the antioxidant effects of exosomal miR-23b compared with those in the exo**NS group (Figure 10C–E).

**BMSC-exosomal miR-23b alleviates NLRP3 inflammasome-mediated pyroptosis by regulating PTEN**

The inhibition of NLRP3 inflammasome components and pyroptosis was reversed by PTEN overexpression (Figure 11A–D). Moreover, exo**NS administration mitigated co-cultured neuronal cell death, which was increased by PTEN overexpression (Figure 11E and F). PTEN was suggested to facilitate the activation of NLRP3 inflammasome by binding with NLRP3 (Huang et al., 2020). We detected the binding of PTEN with NLRP3 after exo**NS administration using co-immunoprecipitation tests and found that overexpressing miR-23b could significantly reduce endogenous PTEN binding to NLRP3 compared with that in the hemin group (Figure 11G).
Exosomal miR-23b attenuates oxidative stress by regulating the GSDMD-N inhibitor.

Exosomal miR-23b negatively regulated PTEN in BV2 cells. (A) Predicted binding between PTEN and miR-23b. (B) Luciferase report assays confirmed binding between miR-23b and PTEN. (C) Relative PTEN mRNA levels after transfection with the mimic and inhibitor. (D) PTEN protein expression after transfection with the mimic and inhibitor. Data are shown as the mean ± SD (n = 3). **P < 0.01 (Student’s t-test). Inhibitors: transfected with miR-23b inhibitors; mimics: transfected with miR-23b mimics; miR-NC: transfected with miR-NC; ns: not significant; PTEN: phosphatase and tensin homolog deleted on chromosome 10.

Figure 8 | Exosomal miR-23b alleviates NLRP3 inflammasome-mediated pyroptosis in microglia BV2 cells and protects neuronal cells in vitro.

(A, B) Expression and data analysis of pyroptosis-related proteins in different groups of microglia BV2 cells. (C, D) IL-1β (C) and IL-18 (D) levels in the BV2 cellular supernatant of microglia BV2 cells were evaluated by ELISA. (E) Cell viabilities of hippocampal neuronal HT22 cells by CCK8 tests. (F) Cell death rates of HT22 cells by LDH assays. Data are shown as the mean ± SD (n = 6). **P < 0.01 vs. PBS group; *P < 0.05, **P < 0.01, vs. hemin group; #P < 0.05, ##P < 0.01, vs. exo−miR group (one-way analysis of variance followed by Newman-Keuls post hoc analysis). PBS group: Control; Hemin group: treated with 60 μM hemin for 24 hours; exo−miR group: treated with miR-NC transfected BMSC-exosomes after hemin stimulation; exo+miR group: treated with miR-23b transfected BMSC-exosomes after hemin stimulation. BMSC: Bone marrow mesenchymal stem cell; CCK8: cell counting kit-8; ELISA: enzyme linked immunosorbent assay; GSSG: oxidized glutathione disulfide; HO-1: heme oxygenase-1; MDA: malondialdehyde; NLRP3: NOD-like receptor family pyrin domain containing 3; PBS: phosphate-buffered saline.

Figure 9 | miR-23b negatively regulated PTEN in BV2 cells.

(A) Western blot bands for PTEN, Nrf2 (cytoplasmic and nuclear), and HO-1 proteins in microglia BV2 cells from different groups. (B) Quantitative analysis of PTEN, Nrf2, (cytoplasmic and nuclear), and HO-1. (C) MDA levels were measured by echomycronal kits. (D) SOD levels were measured by echomycronal kits. (E) GSH/GSSG ratio was calculated on the basis of the GSH and GSSG levels which evaluated by echomycronal kits. Data are shown as the mean ± SD (n = 6). **P < 0.01, *P < 0.05, vs. hemin group; #P < 0.05, ##P < 0.01, vs. exo−miR group (one-way analysis of variance followed by Newman-Keuls post hoc analysis). Hemin: Treated with 60 μM hemin for 24 hours; exo+PTEN: group treated with miR-23b transfected BMSC-exosomes after hemin stimulation; exo+PTEN: treated with pcdNA3.1-PTEN plasmids and miR-23b transfected BMSC-exosomes after hemin stimulation. BMSC: Bone marrow mesenchymal stem cell; GSH: reduced glutathione; GSSG: oxidized glutathione disulfide; HO-1: heme oxygenase-1; MDA: malondialdehyde; Nf2: nuclear factor erythroid-2-related factor 2; PTEN: phosphatase and tensin homolog deleted on chromosome 10; SOD: superoxide dismutase.

Figure 10 | BMSC-exosomal miR-23b attenuates oxidative stress by regulating the PTEN/Nrf2 pathway in microglia BV2 cells in vitro.

(A) Western blot bands for PTEN, Nrf2 (cytoplasmic and nuclear), and HO-1 proteins in microglia BV2 cells from different groups. (B) Quantitative analysis of PTEN, Nrf2, (cytoplasmic and nuclear), and HO-1. (C) MDA levels were measured by echomycronal kits. (D) SOD levels were measured by echomycronal kits. (E) GSH/GSSG ratio was calculated on the basis of the GSH and GSSG levels which evaluated by echomycronal kits. Data are shown as the mean ± SD (n = 6). **P < 0.01, *P < 0.05, vs. hemin group; #P < 0.05, ##P < 0.01, vs. exo−miR group (one-way analysis of variance followed by Newman-Keuls post hoc analysis). Hemin: Treated with 60 μM hemin for 24 hours; exo+PTEN: group treated with miR-23b transfected BMSC-exosomes after hemin stimulation; exo+PTEN: treated with pcdNA3.1-PTEN plasmids and miR-23b transfected BMSC-exosomes after hemin stimulation. BMSC: Bone marrow mesenchymal stem cell; GSH: reduced glutathione; GSSG: oxidized glutathione disulfide; HO-1: heme oxygenase-1; MDA: malondialdehyde; Nf2: nuclear factor erythroid-2-related factor 2; PTEN: phosphatase and tensin homolog deleted on chromosome 10; SOD: superoxide dismutase.
ICH is a serious type of stroke (Ren et al., 2020; Tschoe et al., 2020; Liu et al., 2022). No known major mechanistic roles of miR-23b in NLRP3 inflammasome-mediated pyroptosis, which induces inflammatory cascades and cell death (Luo et al., 2019; Xiao et al., 2020). Oxidative stress, caused by ROS accumulation, further aggravates the redox imbalance and acts as a mediator of NLRP3 (Cheng et al., 2019; Shi et al., 2021). Many clinical trials of exosomes for brain injuries have been conducted (clinicaltrials.gov). A recent prospective observational cohort study focused on using circulating exosomes to make an early diagnosis and to evaluate the prognosis of ICH patients (Shi et al., 2021). It was evident that exosomes could play in translational medicine in the future. Thus, an in-depth exploration of the therapeutic mechanisms of exosomes to provide a solid theoretical basis for clinical translation is urgently needed. We have reported that BMSC-exosomes carrying miR-23b inhibited oxidative stress and pyroptosis and alleviated brain inflammation and edema, thereby promoting behavioral recovery in rats with ICH. We also demonstrated that exosomal miR-23b inhibited NLRP3 inflammasome signaling and exerted antioxidant activities and inhibit cell pyroptosis by suppressing NLRP3 inflammasome activation via targeting PTEN. Collectively, our research suggests that exosomal miR-23b derived from BMSCs plays a neuroprotective role in ICH.

Because free biomedical nanoparticle levels may be reduced by non-specific intra- and extra-cellular interaction in tissues after in vivo delivery (González-Nieto et al., 2020), carriers that can target injured organs, such as BMSCs or exosomes, seem to be more efficient. Compared with BMSCs, exosomes harvested from the naive BMSCs showed therapeutic effects that were consistent with those from BMSCs with a lower risk of embolism formation and tumorigenicity (Wang et al., 2012; Xin et al., 2013). Thus, we administered BMSC-exosomes to ICH rats using tail vein injections. On the basis of our previous data, which showed that miR-23b was downregulated in ICH rats (Zhu et al., 2017; Wang et al., 2019), we selected miR-23b as a target for our study using tailored exosomes with modified miRNA content to improve function and efficiency. We conducted experiments to substantiate our hypothesis. BMSC-exosomes containing elevated miR-23b could be internalized with the brain host cells, which led to miR-23b elevation in the tissue surrounding the hematoma. Additionally, BMSC-derived exosomes containing miR-23b further attenuated the neurological deficits compared with those of naive exosomes. Because brain edema is an independent risk factor for mortality in ICH (Wu et al., 2017), we observed that exosomal miR-23b improved the functional recovery and reduced ICH-induced edema in this study. In our in vivo study, we confirmed the neuroprotective role of BMSC-exosomes with miR-23b in ICH.

When ICH occurs, the hemoglobin-heme-iron metabolic axis is triggered and contributes to ROS overproduction (Zhu et al., 2021). ROS overproduction is known to aggravate ICH-induced neuroinflammation. Pyroptosis requires inflammation activation, such as in the NLRP3 inflammasome, converts precursor caspase-1 into cleaved caspase-1, and then cleaves the precursor of GSDMD into its downstream pyroptosis protein levels in a ROS-dependent manner, thereby amplifying the inflammatory response (Shi et al., 2017; McKenzie et al., 2020). In this study, we hypothesized that co-cultured neuronal cell death was reduced by exosomal miR-23b, as ROS formation and oxidative stress levels, which was accompanied by further reduction of PTEN expression and hampered the interaction between PTEN and NLRP3, thus mitigating NLRP3 inflammasome-mediated pyroptosis. PTEN overexpression could further reverse the inhibition of pyroptosis by miR-23b. Thus, exosomal miR-23b may ameliorate cell pyroptosis and neuroinflammation in ICH by inhibiting NLRP3 inflammasome activation by targeting PTEN.

However, our study had some limitations. Our study investigated whether exosomal miR-23b alleviates the oxidative stress and pyroptosis in ICH by targeting PTEN, but other mechanisms by which exosomal miR-23b exerts its protective effects remain unclear. Evidence shows that mitochondrial dysfunction can promote excessive ROS generation to increase total cellular oxidative stress (Cacialli et al., 2021). Because this study mainly focused on exosomal miR-23b for total intracereolar ROS, further investigation of the involvement of mitochondrial ROS with exosomal miR-23b is warranted in the future study. Furthermore, our research clarified the mechanisms by which exosomal miR-23b regulates NLRP3/caspase-1-dependent pyroptosis, but whether other activated caspase (caspase-4/5/11) activation is involved in pyroptosis remains unknown and needs to be investigated in the future.

In conclusion, this study clarified that BMSC-exosomal miR-23b attenuated oxidative stress and pyroptosis, thereby alleviating neuroinflammation and exerting neuroprotection in ICH. PTEN may serve as a target gene to mediate the antioxidant activity and the anti-inflammatory effect of miR-23b via regulating the Nrf2 signaling pathway and NLRP3 inflammasome activation. Our study demonstrates that ICH may be treated with exosomes derived from BMSCs plays a neuroprotective role in ICH.

Conflict of interest: The authors declare no conflict of interests.

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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Additional file: Additional Table 1: The sequences of primers in this study.

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### Additional Table 1 The sequences of primers in this study

| Name             | Sequences (5'-3')                       |
|------------------|-----------------------------------------|
| miR-23b-3p       | Forward: GCGGTCATCACATTTGCCAG           |
|                  | Reverse: TATGTTGTTTCAGAATCCCTCAC        |
| U6               | Forward: GGAACGATACAGAAGATATTAGC        |
|                  | Reverse: TGGAACGCTCCACGAATTGGCG         |
| mmu-PTEN         | Forward: GGAAAGGGACGGACTGGTGATATG       |
|                  | Reverse: CGCCTCTGAAGGAATTGTGAC          |
| mmu-β-actin      | Forward: GTGCTATTTGCTCTAGACTTCG         |
|                  | Reverse: ATGCCACAGATTCCATAAC            |
| rno-NLRP3        | Forward: GAGCTGAGCCTCAGTGAATG          |
|                  | Reverse: AGAACCATTGCCAGAATGGGACACATACC |
| rno-Caspase-1    | Forward: AAACACCCACTCGTAGCCTGAATGC     |
|                  | Reverse: AGGTCACACATACGCTCCAGACTCTC    |
| rno-GSDMD        | Forward: CAGCAGCAGCATTGAGTACCCGAATGC  |
|                  | Reverse: CCTCCAGAGCCTTATGAGCCGATAG     |
| rno-β-actin      | Forward: TCAGGTCATCAGCTTCCAGGCAAT      |
|                  | Reverse: AAAGAAAGGGTGTAAACGCA          |

PTEN: phosphatase and tensin homolog deleted on chromosome 10; NLRP3: Nod-like receptor family pyrin domain containing 3; GSDMD: gasdermin D.