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Microglia-derived iron-overloaded exosomes induce neuronal ferroptosis and aggravate neurological impairment after subarachnoid hemorrhage

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Abstract

Subarachnoid hemorrhage (SAH) is a devastating stroke subtype often leading to poor neurological outcomes. Iron homeostasis imbalance is a key contributor to cognitive dysfunction in neurological diseases. Extracellular vesicles, including exosomes (EXs), are crucial mediators of intercellular communication. This study investigated the role of EXs in post-SAH iron metabolism and neurological impairment. We isolated EXs from various neural cells (microglia, astrocytes, endothelial cells, neurons; $n=4-6$ independent isolations) in vitro after SAH mimicked by oxyhemoglobin (OxyHb) or hemin. We found that microglial EXs (MC-EXs) were significantly enriched in iron and potently impaired neuronal viability. Using specific inhibitors and fluorescence imaging, we demonstrated that neurons internalize MC-EXs primarily via dynamin-dependent, clathrin-, caveolae-, and lipid raft-mediated endocytosis. Combining transcriptomic analysis with in vivo and in vitro SAH models, we discovered that iron-overloaded MC-EXs induce neuronal ferroptosis. In mice, intranasal administration of MC-EXs (10^9 particles/day for 3 days, $n=10$ /group) exacerbated SAH-induced motor, sensory, and cognitive deficits. Bioinformatic analysis and experimental validation (including C3 siRNA knockdown) identified the complement C3/C5/NF- κ B pathway as a key molecular mechanism through which iron-overloaded MC-EXs trigger ferroptosis. This research provides evidence for a novel EX-mediated mechanism for iron toxicity in SAH, highlighting MC-EXs and the C3/C5/NF- κ B axis as potential therapeutic targets.

Keywords Exosomes, Extracellular vesicles, Microglia, Neurons, Ferroptosis, subarachnoid hemorrhage

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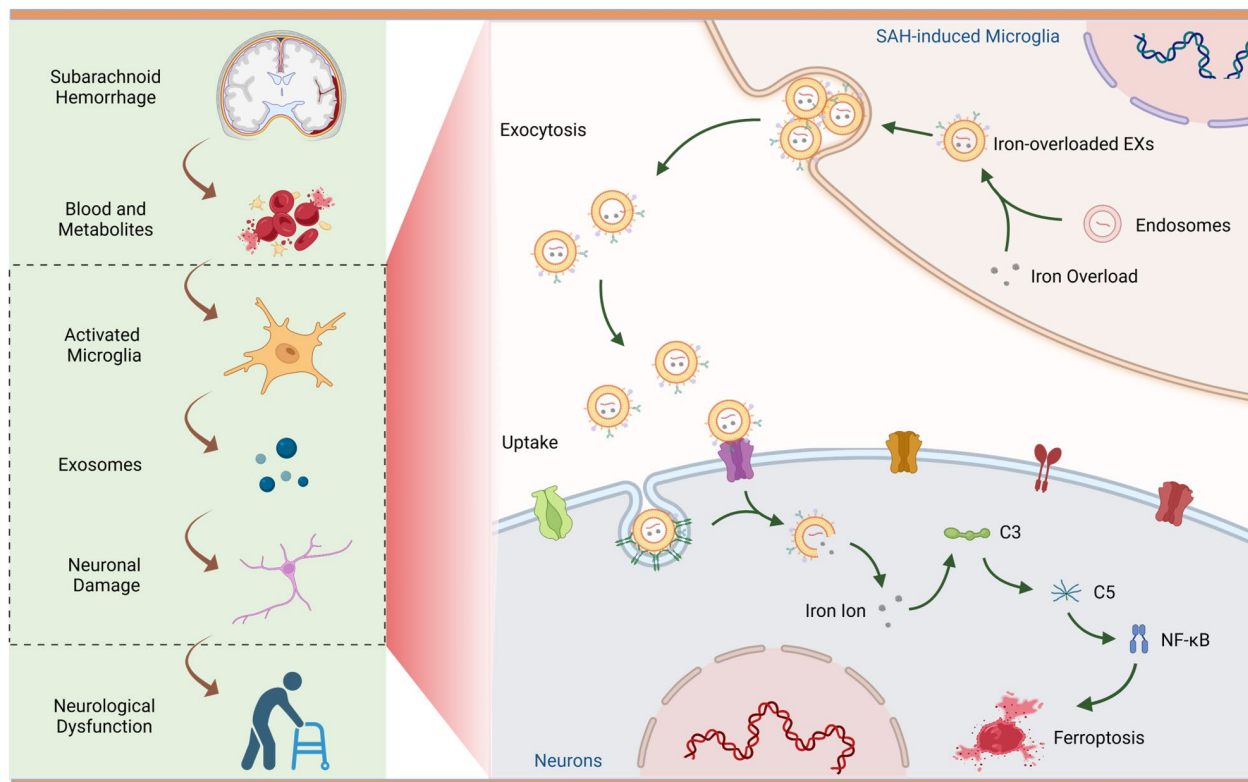
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Graphical Abstract



Introduction

Subarachnoid hemorrhage (SAH) typically arises from a ruptured cerebral aneurysm, accounts for ~ 5% of strokes, and contributes to significant morbidity and mortality rates globally [1]. About 50% of patients who survive SAH suffer major cognitive deficits or neurological sequelae precluding their return to work and daily activities [2]. Long-term neurological dysfunction severely impacts quality of life and imposes a substantial burden on both patients and health care systems [3]. Iron dysmetabolism in the central nervous system (CNS), especially iron overload, is considered a key factor driving the progression of cognitive decline in neurodegenerative diseases [4, 5]. Iron deposition in the cerebrospinal fluid and brain parenchyma also increases after SAH due to blood entering the subarachnoid space. Emerging approaches, such as iron chelation and genetic risk profiling, hold promise for mitigating cognitive impairments [3, 6]. Therefore, it is of great importance to clarify the mechanism underlying iron dysmetabolism in long-term neurological damage after SAH.

As a subset of extracellular vesicles (EVs) ranging in diameter from 30 to 150 nm, exosomes (EXs) have attracted interest due to their potential diagnostic and therapeutic applications in stroke. Numerous studies

confirmed that EXs can mediate cell–cell/tissue communication by delivering cargo [7, 8]. Following brain injury, the functions of CNS cells become dysregulated, leading to the release of EVs that affect surrounding cells. For example, EVs from neurons and astrocytes can modulate the polarization of microglia. Macrophage-derived foam cells within atherosclerotic plaques secrete EXs with overexpression of microRNA (miR)–30c-2-3p and induce brain damage during ischemic stroke [9]. Astrocyte-derived EXs cultured in a hypoxic environment enhance neuronal defenses against oxidative and ischemic stress [10]. In intracerebral hemorrhage (ICH), the EVs secreted by activated microglia are enriched with miR-383-3p, promote necroptosis in neurons, and exacerbate neuronal death following ICH [11]. In the human induced pluripotent stem cell-derived tri-culture system, researchers found microglia have the greatest response to iron exposure and sequester the greatest amount per cell, lead to increased iron deposition and cell death in neurons, and promote neurodegenerative damage [12]. In SAH, the cascade regulation mechanism of various types of nerve cells through EXs, especially the research on the role of iron ion metabolism, remains to be elucidated.

In this study, we examined the iron ion content of EXs released by four different types of nerve cells after SAH

and their effects on healthy neurons, screened for the EXs that can induce neuronal damage by transducing iron ions, and elucidated their uptake mechanism in neurons. With the assistance of transcriptomic and bioinformatic analysis, we predicted the pathological phenotype and molecular mechanism occurring in neurons and then combined our findings with SAH in *in vivo* and *in vitro* models to verify the predicted hypotheses and explored the effects of the above-mentioned iron-overloaded EXs on various neurological functions after SAH (Fig. 1A). The above series of explorations have provide compelling evidence for EXs are involved in the pathological process

of SAH, and explain the potential donor cells, recipient cells, and regulatory mechanisms involved, providing direction for the research of new and effective treatment options in the future. Based on the above, we hypothesized that microglia-derived exosomes (MC-EXs), by serving as vehicles for intercellular iron transfer, induce neuronal ferroptosis and contribute to long-term neurological impairments after SAH, and that this process is mediated through the activation of the complement C3/C5/NF- κ B signaling pathway.

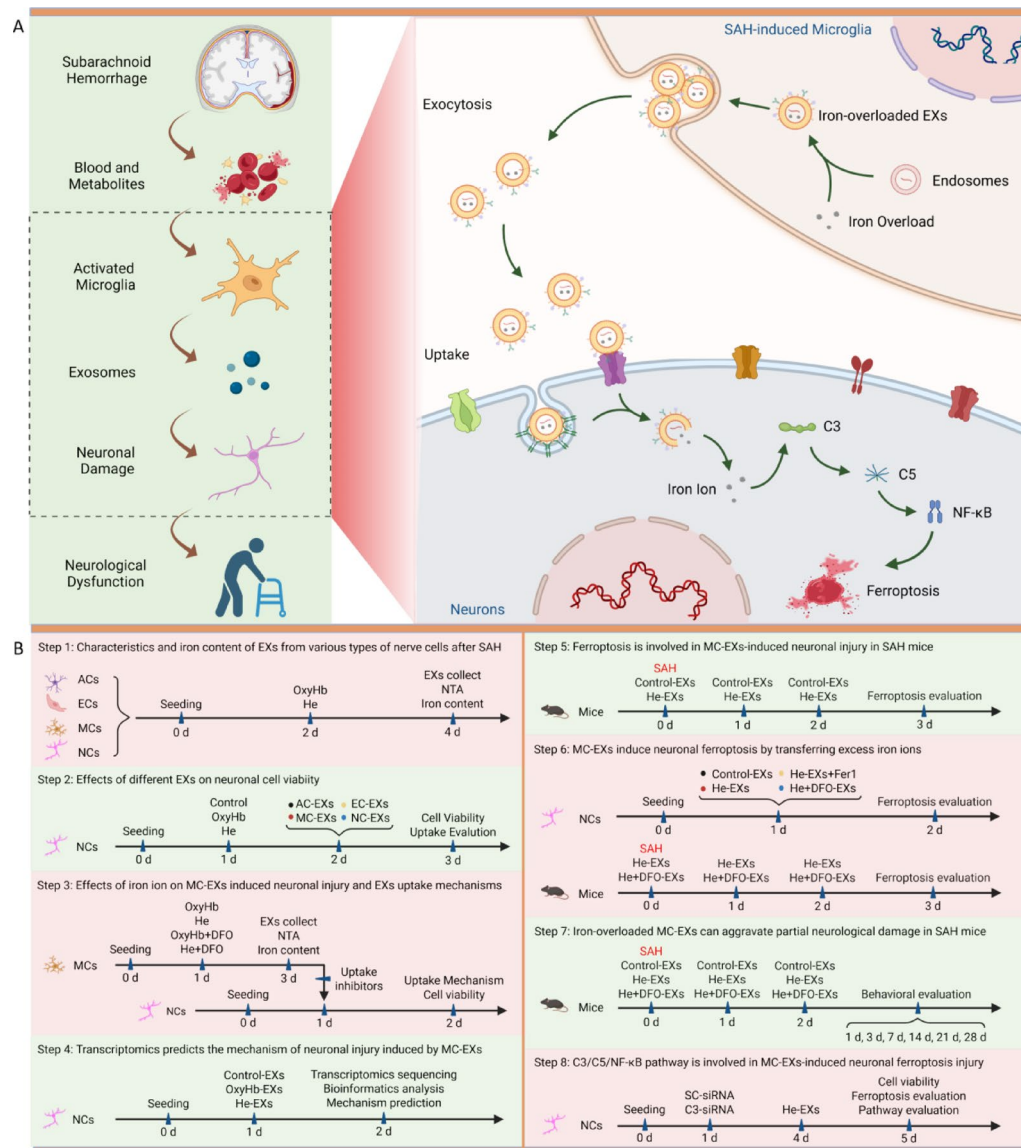


Fig. 1 Summarizing schema of research content and process. **(A)** This study mainly screened and verified that EXs released by microglia after subarachnoid hemorrhage can induce ferroptosis-related damage to neurons by transducing iron ions and can further exacerbate a range of neurological deficits in experimental animals. At the same time, the mechanism by which neurons take up MC-EXs and the molecular mechanisms that may be involved in associated damage were explored; **(B)** Experimental protocols. OxyHb, oxyhemoglobin; EXs, exosomes; WB, western blot; He, Hemin; NTA, nanoparticle tracking analysis; ACs, astrocytes; ECs, endothelial cells; MCs, microglia; NCs, neurons; DFO, Deferoxamine

Materials and methods

Ethics statement

C57BL/6 mice (8–12 weeks, 24–28 g in weight) were used in this study. All animal experiments were approved by the Institutional Animal Care and Use Committee (Project number: 760) of Marshall University, Huntington, WV, USA. All animals were housed under controlled environmental conditions (temperature: 22 ± 2 °C, humidity: 50–60%) with free access to standard laboratory chow and water. Animals were acclimatized to the housing environment for at least 3 days prior to the start of the experiments to minimize stress and ensure physiological stability.

Experimental design and blinding

As outlined in Fig. 1B, we first isolated exosomes (EXs) from four neural cell types under two in vitro SAH model conditions (OxyHb and hemin treatment). We characterized these EXs for concentration, size, and iron content, and assessed their effects on neuronal viability to identify the most neurotoxic EX population. The uptake mechanisms of microglial EXs (MC-EXs) by neurons were then probed using specific endocytic inhibitors. Transcriptomic analysis of MC-EX-treated neurons was performed to uncover underlying injury pathways. By comparing the effects of MC-EXs with those of the ferroptosis inhibitor Ferrostatin-1 (Fer-1) and the iron chelator deferoxamine (DFO), we confirmed that MC-EXs induce neuronal ferroptosis via iron delivery in both in vitro and in vivo SAH models. To evaluate the impact of SAH-induced MC-EXs on neurological function, SAH model mice received intranasal administration of MC-EXs (10^9 particles/day for 3 days) and underwent a battery of behavioral tests assessing sensorimotor function, muscle strength, emotion, and cognition at multiple time points. Bioinformatic analysis using ferroptosis-related and STRING databases predicted involved signaling pathways, which were subsequently validated through C3 siRNA knockdown experiments.

To minimize bias, a randomized, single-blind design was employed for all animal studies. After SAH induction, Researcher A (blinded to subsequent procedures) randomized surviving mice into experimental groups using a computer-generated scheme based on baseline neurological scores. Unique identifiers (e.g., M001, M002) concealed group allocation from Researcher B, who performed all behavioral tests and data collection. Only after all raw data were recorded and verified did Researcher A unblind the identifiers for Researcher C to perform statistical analysis.

Cell culture

Four human cell lines were purchased from ATCC (MD, USA): neuroblastoma cell line (SH-SY5Y), microglial cell

lines (HMC3), astrocyte cell line (CCF-STTG1), cerebral microvascular endothelial cell line (HBEC-5i). All cell types were cultured in their respective complete culture medium in an atmosphere of 5% CO₂ at 37 °C with 10% fetal bovine serum (HyClone, PA, USA), 100 IU/ml penicillin and 100 mg/ml streptomycin (HyClone, PA, USA) included in all complete culture medium. The medium was changed every 3 days. Passages 10–16 of the cells were used in the present study.

In vitro SAH model

To construct in vitro SAH models, oxyhemoglobin (OxyHb) and hemin were used to mimic the injury that occurs to the cells in the CNS after SAH [13, 14]. In brief, the above four types of nerve cells were seeded in culture plates and cultured with complete medium for 24 h. Then, the medium was removed, and the cells were exposed to 10 μ M OxyHb (Sigma-Aldrich, MO, USA) or hemin (Sigma-Aldrich, MO, USA) in complete medium for 24 h (h). The dose and time point of OxyHb were selected according to our previous study [13]. The dose of hemin was based on the IC₅₀ value obtained from CCK8 experiments.

Exosome extraction and purify control

The EXs were collected from the medium of different cells according to our previously reported method [13]. Briefly, EPCs were cultured under standard conditions until 80% confluence, followed by washing with sterile PBS to remove residual serum proteins and FBS-derived exosomes. The growth medium was then replaced with serum-free media (SFM), and the cells were incubated for 48 h to allow for exosome secretion into the conditioned media. The collected SFM was processed immediately or stored at 4 °C for short periods (≤ 24 h), or at -80 °C for longer storage. To remove cells and large debris, the SFM was centrifuged at $300 \times g$ for 10 min at 4 °C, and the supernatant was transferred to ultracentrifuge tubes. Next, apoptotic bodies and larger particles were removed by centrifugation at $2,000 \times g$ for 20 min at 4 °C, followed by another centrifugation at $20,000 \times g$ for 70 min at 4 °C to remove larger vesicles and organelles. The pre-cleared supernatant was then subjected to ultracentrifugation at $170,000 \times g$ for 90 min at 4 °C to pellet the exosomes. After carefully aspirating the supernatant, the exosome pellet was resuspended in 500 μ L of cold, sterile PBS, using gentle pipetting to avoid bubble formation.

Nanoparticle tracking analysis and transmission electron microscopy

For exosome characterization, nanoparticle tracking analysis (NTA) was performed. The exosome sample was diluted in sterile, particle-free PBS to an appropriate concentration for NTA (typically 1:140, with 5 μ L

of exosomes in 695 μL of PBS). The diluted sample was mixed gently, and the NTA instrument was powered on. The fluidic system was cleaned by flushing with particle-free water, and the sample chamber was primed with particle-free PBS to check for background contamination. Data acquisition was performed by loading the diluted exosome sample into the sample chamber and setting the camera level (typically between 12 and 16) and detection threshold (typically between 3 and 5) to optimize particle visualization. Three to five 60-second videos were recorded, ensuring the particle count per frame was within the optimal range. The videos were processed using the NTA software, which generated two key results: (1) a size distribution profile indicating the modal and mean hydrodynamic diameter of the exosome population, with a typical peak between 50 and 150 nm, and (2) the particle concentration (particles/mL), calculated based on the dilution factor.

PKH 26 staining and uptake assessment of EXs

The EXs were labeled with PKH26 (Sigma-Aldrich, MO, USA) according to our previously published method [13, 15], and co-incubated with SH-SY5Y cells for 24 h. After incubation, cells were fixed with 4% paraformaldehyde (PFA) and stained with 4',6-diamidino-2-phenylindole (DAPI). To evaluate the uptake of EXs in mice, brain tissues were obtained at 6 and 24 h after intranasal administration of PKH26-labeled EXs, then routinely fixed, dehydrated, embedded in optimal cutting temperature compound (OCT), and sectioned at 10 μm thickness. Cryosections were brought to room temperature, washed with PBS, and permeabilized with 0.3% Triton X-100 for 15 min. After blocking with 5% normal goat serum for 1 h, sections were incubated overnight at 4 $^{\circ}\text{C}$ with anti-neuron-specific nuclear protein (NeuN) primary antibody (1:500, Millipore, MA, USA). Following PBS washes, sections were incubated with Alexa Fluor 594 secondary antibody (1:500, Thermo Fisher, MA, USA) for 1 h at room temperature in the dark. Nuclei were counterstained with DAPI (1 $\mu\text{g}/\text{mL}$) for 5 min, and slides were mounted with antifade medium. Fluorescence was observed using the EVOS cell imaging system and analyzed using Image J software (Image J, NIH, USA).

To assess uptake mechanisms, neurons grown in 6-well plates were pre-treated with inhibitors as previous reported [13, 15, 16]: Dynasore (80 μM), Genistein (200 μM), Pitstop 2 (10 μM), methyl- β -cyclodextrin (M β CD; 10 mM) and LY294002 (5 μM) for 25 min, then washed once with PBS and co-incubated with MC-EXs labeled with PKH 26 in complete medium for 24 h. Fluorescence imaging and analysis were performed as above.

Cell viability and cytotoxicity assays

Cell viability was assessed using Cell Counting Kit-8 (CCK-8, Sigma-Aldrich, MO, USA), according to the manufacturer's instructions. Briefly, neurons were seeded into 96-well plates and incubated overnight. The following day, cells were treated with varying concentrations of the tested EXs or compounds and incubated for 24 h. Then 10 μL of CCK-8 reagent was added to each well and incubated at 37 $^{\circ}\text{C}$ for 4 h. The results were measured on a microplate reader (BioTek, VT, USA). The optical density (OD) was read at 450 nm, and each group had triplicate wells, blank wells containing medium and CCK-8 without cells. The final concentration of Hemin in different cells refers to the IC_{50} value.

A Cytotoxicity Detection Kit (lactate dehydrogenase (LDH), Sigma-Aldrich, MO, USA) was used to assess cytotoxicity. Cells were seeded in 96-well plates and exposed to different groups of MC-EXs treatments. Then 50 μL of culture supernatant was transferred to a new plate and mixed with 50 μL of LDH reaction reagent, incubated at room temperature for 30 min in the dark, and absorbance was measured at 490 nm and 620 nm using the microplate reader. Spontaneous and maximum LDH release controls were included. Cytotoxicity was calculated using the formula provided in the manufacturer's instructions.

Transcriptomic sequencing and bioinformatics analysis

Transcriptomic sequencing (RNA-seq) was used to detect RNA changes in neurons after exposure to MC-EXs. After preparing sequencing libraries with adapters, the samples were sequenced using next-generation sequencing platforms. The raw data were then processed to remove low-quality reads, aligned to a reference genome, and quantified to determine gene expression levels. This method enabled analysis of gene activity and cellular functions at the transcript level.

Bioinformatic analysis was performed on the transcriptomics results. Differentially expressed genes (DEGs) were identified using the R package limma ($|\text{Log}_2\text{FC}| \geq 0$, $p \leq 0.05$), and volcano plots were used to visualize gene expression changes. Heatmaps showed the top 20 up- and down-regulated genes. KEGG and GO enrichment analyses were conducted to identify commonly affected pathways and biological processes. GSEA was performed to further explore pathway enrichment. Ferroptosis-related genes were identified by overlapping DEGs with the GSE197104 dataset. Additionally, the GSE184917 dataset was analyzed to examine the effects of C3 treatment after stroke. Finally, the STRING database (version 12.0) retrieved from the literature was used to predict the downstream molecules of C3.

In vivo SAH model

The internal carotid artery puncture method was used to induce SAH in mice [17]. Briefly, a midline ventral neck incision was made to expose and ligate the left common carotid artery, followed by ligation and cutting of the external carotid artery. A small dorsal incision was made in the left common carotid artery to insert a 6 – 0 nylon filament, which was advanced into the internal carotid artery about 9–12 mm to the middle cerebral artery. The insertion site was ligated. The filament was advanced until a sudden rise in intracranial pressure occurred, then withdrawn, and the external carotid artery was ligated to prevent bleeding. The skin was closed with 4 – 0 absorbable sutures. Mice were given buprenorphine subcutaneously postoperatively, monitored during recovery and maintained at 37.0 °C with a homeothermic heating pad. Animals in Sham group underwent the same surgical procedures without vessel perforation.

Neurological assessment

On days 1, 3, 7, 14, and 21 after SAH induction, sensorimotor function in mice was assessed using the adhesive removal test (ART) and rotarod test (RT), and limb strength was assessed using the grip strength test (GT). On days 3, 7, 14, 21, and 28 after SAH induction, cognitive function was assessed using the novel object recognition test (NORT) and Y-Maze test, and anxiety-like behavior and locomotor activity was assessed using the open field test (OFT). For detailed test methodology, please refer to the supplementary methods section. Before starting the test, the experimental animals were placed in the experimental room for 30 min of pre-adaptation. The chamber or apparatus was thoroughly cleaned with 70% ethanol between tests to eliminate olfactory cues.

Iron quantification

Iron quantification was performed using the Iron Assay Kit (MAK472). Prior to analysis, samples were processed by centrifugation or filtration if turbidity was present. A 50 µL aliquot of each sample was transferred to a clear, flat-bottom 96-well plate, and a blank sample was prepared. A standard curve was established by diluting a 10 mg/dL iron standard solution with purified water to generate a series of iron standard solutions at varying concentrations. A 50 µL volume of each standard solution was added to individual wells. The working reagent was prepared by mixing Reagent A, Reagent B, and Reagent C. Subsequently, 200 µL of the working reagent was added to each sample and standard well, with the blank sample receiving only Reagent A. Samples and standards were incubated at room temperature for 40 min. After incubation, OD at 590 nm was measured using a spectrophotometer. The OD difference (Δ OD)

for each sample was calculated by subtracting the blank value. The standard curve was plotted, and linear regression was performed to derive the slope, which was then used to calculate the iron concentration in the samples.

Iron content assays

The iron ion content in neurons and EXs in vitro was determined using an Iron Assay Kit (Sigma-Aldrich, MO, USA). According to the product manual, 50 µL of each sample was added to a 96-well plate. A standard curve was prepared using serial dilutions of an iron standard, then 200 µL of working reagent were added to each well. The plate was gently mixed, incubated at room temperature for 40 min, and OD was measured at 590 nm. All samples and standards were run in duplicate.

The iron content in brain tissue was assessed by iron staining of paraffin sections. The sections were deparaffinized and hydrated with deionized water. Slides were incubated in Working Iron Stain Solution (Sigma-Aldrich, MO, USA) for 10 min, then rinsed in deionized water, and subsequently stained with Working Pararosaniline Solution for 2 min and rinsed in deionized water. Slides were then rapidly dehydrated through graded alcohol and xylene and mounted for microscopic analysis.

SiRNA transfection

To inhibit the expression of complement C3 in neurons, we transfected cells with control siRNA and C3 siRNA (Santa Cruz, CA, USA), respectively. Cells were seeded in 6-well plates at 2×10^5 cells per well in antibiotic-free growth medium and incubated at 37 °C until 60–80% confluence. For transfection, siRNA (2/4/6 µl) was diluted in 100 µl serum-free Transfection Medium (Solution A), and siRNA Transfection Reagent (5 µl) was diluted in 100 µl Transfection Medium (Solution B). Solution A was added to Solution B, mixed gently, and incubated for 30 min at room temperature to form complexes. Cells were washed with Transfection Medium, then 0.8 ml of the siRNA–reagent mixture was added to each well. After 5 h incubation at 37 °C, 1 ml of 2× serum-containing growth medium was added without removing the transfection mixture. Cells were further incubated overnight before replacing the transfection mixture with fresh complete medium. Assays were performed 24 h post-transfection.

Assessment of lipid peroxidation levels

Accumulation of lipid peroxide in neurons was detected with a Image-iT Lipid Peroxidation Kit (Molecular Probes, OR, USA). Cells were seeded in a 6-well plate at a density of 2×10^5 and incubated for 24 h. Then the staining solution (10 µM) was added to the cells and incubated for 30 min at 37 °C. The cells were then harvested and

analyzed with Accur C6 Plus flow cytometry (BD Biosciences, CA, USA).

The malondialdehyde (MDA) content in mouse brain tissue reflects lipid peroxide levels. For detailed procedures, see the Lipid Peroxidation (MDA) Assay Kit manual (Sigma-Aldrich, MO, USA). Briefly, samples were homogenized and centrifuged. The supernatant was reacted with thiobarbituric acid (TBA) at high temperature to form MDA-TBA complexes. Absorbance was measured at 532 nm, and MDA levels were calculated using a standard curve, normalized to protein content.

Transmission electron microscopy (TEM)

Mice were deeply anesthetized and transcardially perfused with ice-cold phosphate-buffered saline (PBS, pH 7.4) followed by a fixative containing 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer (PB). The brain tissue was rapidly dissected into approximately 1-mm³ blocks, immersed in 2.5% glutaraldehyde at 4 °C overnight, rinsed in PB, and post-fixed in 1% osmium tetroxide for 1–2 h at room temperature. After dehydration through a graded ethanol series and propylene oxide, samples were infiltrated with Epon 812 resin and polymerized at 60 °C for 48 h. Ultrathin Sects. (60–80 nm) were cut using an ultramicrotome, mounted on copper grids, and stained with uranyl acetate and lead citrate. Images were acquired using a transmission electron microscope (JEOL; 80–120 kV), with all procedures performed under blinded conditions.

Western blot

Mouse brain tissue, cultured neurons or EXs were homogenized in ice-cold lysis buffer (Thermo Fisher, MA, USA) for protein extraction. Protein concentrations were measured using the BCA Assay (Bio-Rad, CA, USA). Equal amounts of protein were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to 0.45-μm polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with 5% non-fat milk for 1 h at room temperature, then incubated overnight at 4 °C with primary antibodies against CD63 (1:200, Santa Cruz, CA, USA), tumor susceptibility gene 101 (TSG101; 1:1000, Santa Cruz, CA, USA), glutathione peroxidase 4 (GPx4; 1:500, Sigma Aldrich, MO, USA), cyclooxygenase-2 (COX2; 1:500, Abcam, Cambridge, UK), Complement C3 (1:1000, Thermo Fisher, MA, USA), C5 (1:1000, Thermo Fisher, MA, USA), nuclear factor kappa B (NF-κB) p65 (1:1000, Thermo Fisher, MA, USA), and beta-actin (β-actin; 1:4000, Sigma Aldrich, MO, USA). After incubation with horse-radish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature and protein bands were visualized using the Odyssey Infrared Imaging System (LI-COR,

NE, USA). Band intensity was quantified with ImageJ, using β-actin as an internal reference.

Statistics

Data are presented as mean ± standard deviation (SD). A two-tailed Student's *t* test followed by Welch's correction was used for comparison between two groups. A one-way analysis of variance (ANOVA) was used for comparisons among multiple groups. Bonferroni or Dunn's post hoc analyses were used to determine where differences occurred. All longitudinal behavioral data collected over time were analyzed using two-way repeated-measures ANOVA, followed by Bonferroni's post hoc test. Raw RNA-seq reads were aligned to the reference genome and quantified. Differential expression analysis was performed using DESeq2 or edgeR, with genes having adjusted *P* < 0.05 and |log₂ fold change| ≥ 1 considered significant. All analyses were carried out using GraphPad Prism 10.0 Software (GraphPad Software, LaJolla, CA, USA). The criterion for statistical significance was *P* < 0.05.

Results

Characteristics of EXs released by various types of nerve cells

We employed two in vitro models of SAH with OxyHb or hemin. For the Hemin model, we applied gradient concentrations of Hemin to astrocytes, endothelial cells, MCs, and neurons. Cell viability was assessed using the CCK-8 assay, and the IC₅₀ value was calculated for each cell type to determine the final working concentration of Hemin (Figure S1 A–D). Then, EXs from the Control group, OxyHb group, and Hemin group were collected for analysis for each of the above four types of cells. In the OxyHb model, NTA analysis showed no significant differences in the concentration or diameter of EXs released by the various cell types (*P* > 0.05; Figure S2A–C). The expression of the EX markers CD63 and TSG101 was more prominent in the EXs compared to their corresponding cell lysates (Figure S2D). In the Hemin model, NTA analysis showed a significant increase in EXs release from astrocytes and neurons compared to the Control group (*P* < 0.05; Figure S2E, F). Additionally, the diameter of neuronal EXs was significantly larger than that of the Control group (*P* < 0.05; Figure S2G). CD63 and TSG101 were detectable in the EXs of all cell groups (Figure S2H).

Iron-overloaded EXs released by microglia after SAH lead to neuronal damage

We then aimed to evaluate the iron content in EXs and their effects on healthy neurons. In the OxyHb model, the iron content in EXs released by astrocytes (*P* < 0.05) and MCs (*P* < 0.01) was significantly higher than that in the Control group (Fig. 2A). Moreover, MC-EXs from

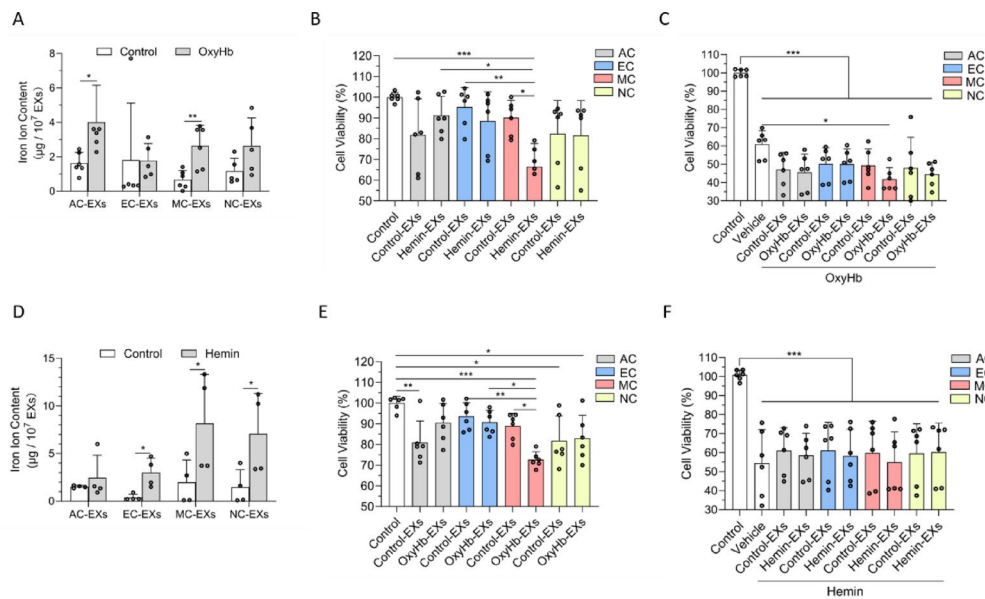


Fig. 2 Microglial exosomes released after SAH induced impairment of healthy neuronal cell viability. **(A)** quantitation of iron ion content in EXs from control or OxyHb-treated cells, $n=4$; **(B)** cell viability of neurons treated with EXs from control or OxyHb-treated cells, $n=6$; **(C)** cell viability of neurons treated with OxyHb plus EXs from control or OxyHb conditioned cells, $n=6$; **(D)** quantitation of iron ion content in EXs from control or Hemin-treated cells, $n=4$; **(E)** cell viability of neurons treated with EXs from control or Hemin-treated cells, $n=6$; **(F)** cell viability of neurons treated with Hemin plus EXs from control or Hemin conditioned cells, $n=6$. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. Statistical method: mean $\pm$ standard deviation (SD), AD: paired t test, BCEF: A one-way analysis of variance (ANOVA)

the OxyHb group significantly reduced the viability of healthy neurons compared to Control EXs ($P<0.05$; Fig. 2B). Additionally, in OxyHb-treated neurons, incubation with MC-EXs further exacerbated cell damage ($P<0.05$ vs. Vehicle; Fig. 2C). In the Hemin model, the iron content in EXs released by MCs and neurons was also significantly elevated compared to the Control group ($P<0.05$; Fig. 2D). MC-EXs from the Hemin group similarly impaired the cell viability of healthy neurons ($P<0.05$ vs. Control EXs; Fig. 2E). However, EXs from each cell group had no significant effect on the viability of neurons already treated with Hemin ($P>0.05$; Fig. 2F). These results suggest that in SAH, the iron content in MC-EXs is significantly increased and contributes to neuronal injury.

Mechanism of MC-EX uptake by neurons

In this experiment, we co-cultured neurons with EXs labeled with PKH26 from different groups of cells and captured fluorescent images for analysis (Fig. 3A). The results showed that neuronal uptake of EXs from both the control and Hemin-treated cell groups was comparable ($P>0.05$, Fig. 3B). To investigate the specific mechanisms underlying the uptake of MC-EXs by neurons, we first examined the morphology of MC-EXs using TEM. The results revealed the characteristic ring- or cup-shaped vesicles with diameters ranging from 30 to 150 nm (Fig. 3C). Next, neurons were pretreated with five small-molecule inhibitors (Dynasore, Genistein, Pitstop

2, M β CD, and LY294002) followed by co-culture with PKH26-labeled MC-EXs. Fluorescence imaging showed that, compared to the Control group, the fluorescence intensity of internalized EXs was significantly reduced in the Dynasore ($P<0.01$), Genistein ($P<0.05$), Pitstop 2 ($P<0.05$), and M β CD ($P<0.001$) groups (Fig. 3D, E). Furthermore, analysis of the fluorescence distribution revealed that the red fluorescence of EXs was primarily localized within the cytoplasm along the long axis of the neurons (Fig. 3F, G). These findings suggest that MC-EXs are internalized by neurons through multiple endocytic pathways, including lipid raft-mediated, dynamin-dependent, clathrin-mediated, and caveolae-mediated endocytosis, and are predominantly localized in the cytoplasm after uptake.

MC-EXs cause neuronal damage in an iron-dependent manner

To determine whether the neuronal damage induced by MC-EXs is iron-dependent, SAH MCs in vitro models were treated with DFO or GW4869 (EXs secretion inhibitor). EXs were then collected from each group. Western blot analysis confirmed the presence of the EX markers CD63 and TSG101 in all groups (Fig. 4A). NTA analysis showed that EXs from the Hemin + DFO group had a significantly smaller average diameter compared to those from the other two groups ($P<0.01$; Fig. 4B, C). In terms of concentration, GW4869 treatment led to a significant

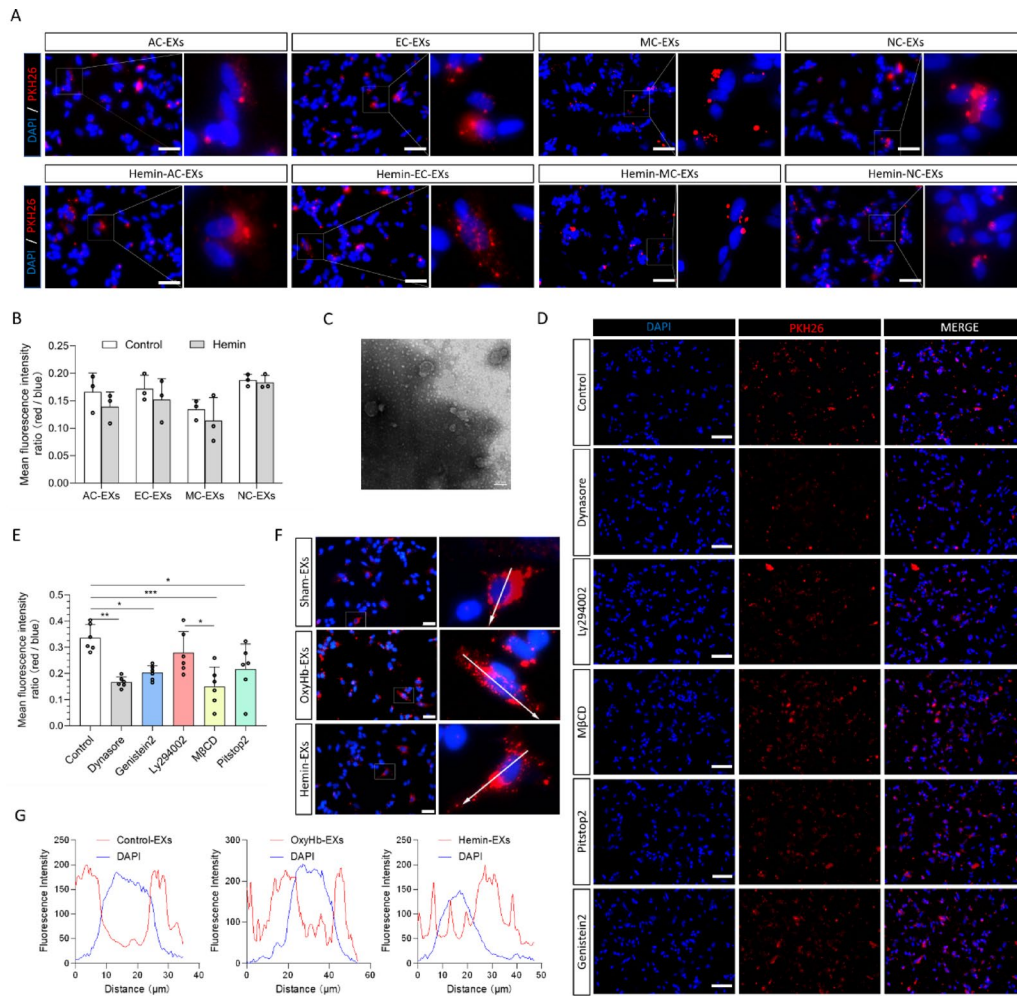


Fig. 3 Mechanism of microglial exosome uptake by neurons after subarachnoid hemorrhage. **(A)** representative images of PKH26-labeled EXs from different cells taken up by neurons, bar = 50 μm ; **(B)** statistical graph of fluorescence intensity of EXs in neurons; **(C)** the representative image of the typical morphology of MC-EXs observed by transmission electron microscopy, bar = 100 nm; **(D)** representative images of PKH26 labeled MC-EXs in neurons of each EXs uptake pathway inhibitor group, bar = 50 μm ; **(E)** statistical graph of EXs fluorescence intensity in each group of neurons, $n = 6$; **(F)** representative images of the distribution of EXs and nuclear fluorescence in neurons, bar = 50 μm ; **(G)** analysis of the distribution of PKH26 and DAPI fluorescence along the long axis of cells. Statistical method: mean $\pm$ standard deviation (SD), B:paired t test, E:A one-way analysis of variance (ANOVA)

reduction in EXs secretion ($P < 0.01$ vs. OxyHb, $P < 0.05$ vs. Hemin; Fig. 4D).

Subsequently, we measured the iron content of EXs in each group. DFO treatment significantly reduced the iron levels in EXs ($P < 0.05$ vs. OxyHb, $P < 0.05$ vs. Hemin), whereas GW4869 treatment unexpectedly led to an increase in iron content ($P < 0.05$ vs. OxyHb, $P < 0.01$ vs. Hemin; Fig. 4E). To assess the functional effects, neurons were co-cultured with EXs from different groups. EXs derived from both SAH model groups (OxyHb and Hemin) significantly increased neuronal cytotoxicity compared to the Control group ($P < 0.05$). However, cytotoxicity was alleviated by DFO pretreatment ($P < 0.05$ vs. OxyHb, $P < 0.05$ vs. Hemin; Fig. 4F). These results demonstrate that chelating iron with DFO during EXs biogenesis successfully generates iron-depleted MC-EXs,

and that these iron-deficient vesicles lose most of their neurotoxic capacity.

We further conducted transcriptome sequencing of neurons treated with EXs from the Control, OxyHb, and Hemin groups. The volcano plot (Fig. 4G) highlights significantly up- and down-regulated genes, and the heat-map displays the top 20 most differentially expressed genes (Fig. 4H). Enrichment analysis showed that these genes were linked to oxidative damage pathways, especially ferroptosis (Fig. 4I, J). GSEA further confirmed significant enrichment of the ferroptosis pathway in neurons exposed to MC-EXs from the model groups ($\text{NES} = 1.51$, $P = 0.02$, Fig. 4K). These findings suggest iron contributes to MC-EX-induced neuronal injury, and ferroptosis may be a key mechanism.

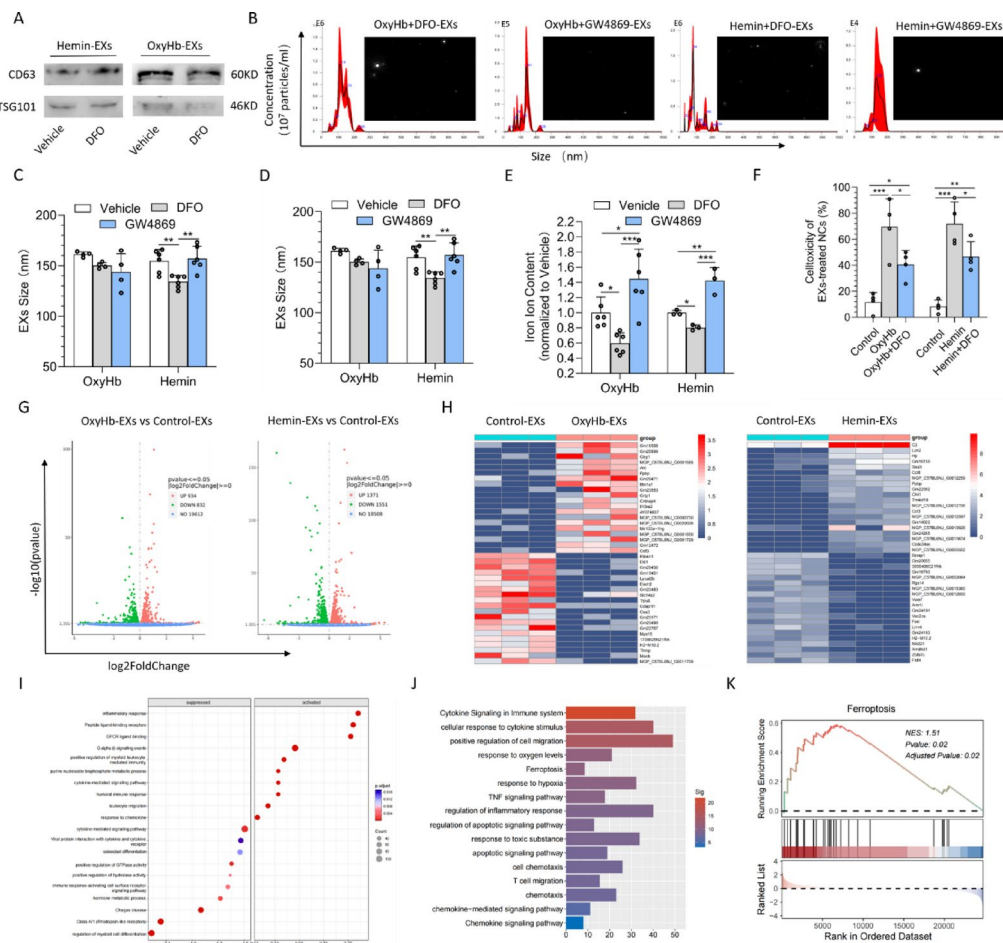


Fig. 4 Iron-overloaded MC-EXs induced neuronal damage by transducing iron ions. **(A)** representative bands of EXs markers CD63 and TSG101; **(B)** representative NTA frames and concentration/size graphs of EXs from DFO- or GW4869-treated MCs; **(C)** statistical chart of EXs concentration, $n=6$; **(D)** statistical chart of EXs size, $n=6$; **(E)** quantitation of iron ion content in EXs from SAH neurons with or without DFO, $n=3/6$ conditioned; **(F)** cytotoxicity of neurons treated with MC-EXs from different groups, $n=4$; **(G)** volcano plots are used to display the differential gene expression between each treatment group (OxyHb-EXs or Hemin-EXs) and the control group; **(H)** heatmaps show the expression levels of the top 20 upregulated and downregulated genes; **(I)** KEGG pathway enrichment analysis shows the commonly activated and suppressed pathways in both treatment groups; **(J)** GO analysis shows the biological pathways commonly enriched by the differentially expressed genes shared between the two treatment groups; **(K)** GSEA analysis demonstrates that genes associated with the ferroptosis pathway are significantly enriched. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Statistical method: mean $\pm$ standard deviation (SD), CDEF: A one-way analysis of variance (ANOVA)

MC-EXs induce neuronal ferroptosis after SAH by transferring iron ions

To further verify the involvement of ferroptosis in MC-EXs-induced neuronal damage following SAH, MC-EXs were isolated from in vitro SAH models and co-cultured with neurons. The two experimental groups were treated with DFO and Fer-1, respectively. Cell viability assays revealed that both Hemin-EXs and OxyHb-EXs significantly reduced neuronal viability ($P < 0.001$). DFO treatment alleviated the cell impairment ($P < 0.01$ vs. Hemin-EXs; $P < 0.05$ vs. OxyHb-EXs). Administration of Fer-1 (10 μ M) also attenuated Hemin-EX-induced neuronal damage ($P < 0.05$; Fig. 5A, B). Furthermore, analysis of ferroptosis-related markers revealed that Hemin-EX treatment significantly decreased GPx4 expression

($P < 0.05$) and increased COX2 expression ($P < 0.05$) in neurons (Fig. 5C–D). The iron staining results showed that DFO treatment reduced the iron-positive area in neurons induced by Hemin-EXs (Fig. 5F). Consistent with this finding, quantitative iron content assays confirmed that Hemin-EXs markedly increased intracellular iron levels ($P < 0.001$), whereas treatment with DFO ($P < 0.01$) or Fer-1 ($P < 0.05$) mitigated the iron accumulation (Fig. 5G). Lipid peroxide staining further revealed that Hemin-EXs induced a significant increase in lipid peroxidation damage in neurons ($P < 0.001$, negative control shown in Figure S3A), which was attenuated by DFO treatment ($P < 0.05$, Fig. 5H and I). Moreover, Fer-1 treatment suppressed the Hemin-EX-induced upregulation of COX2 ($P < 0.05$) and reversed the downregulation of

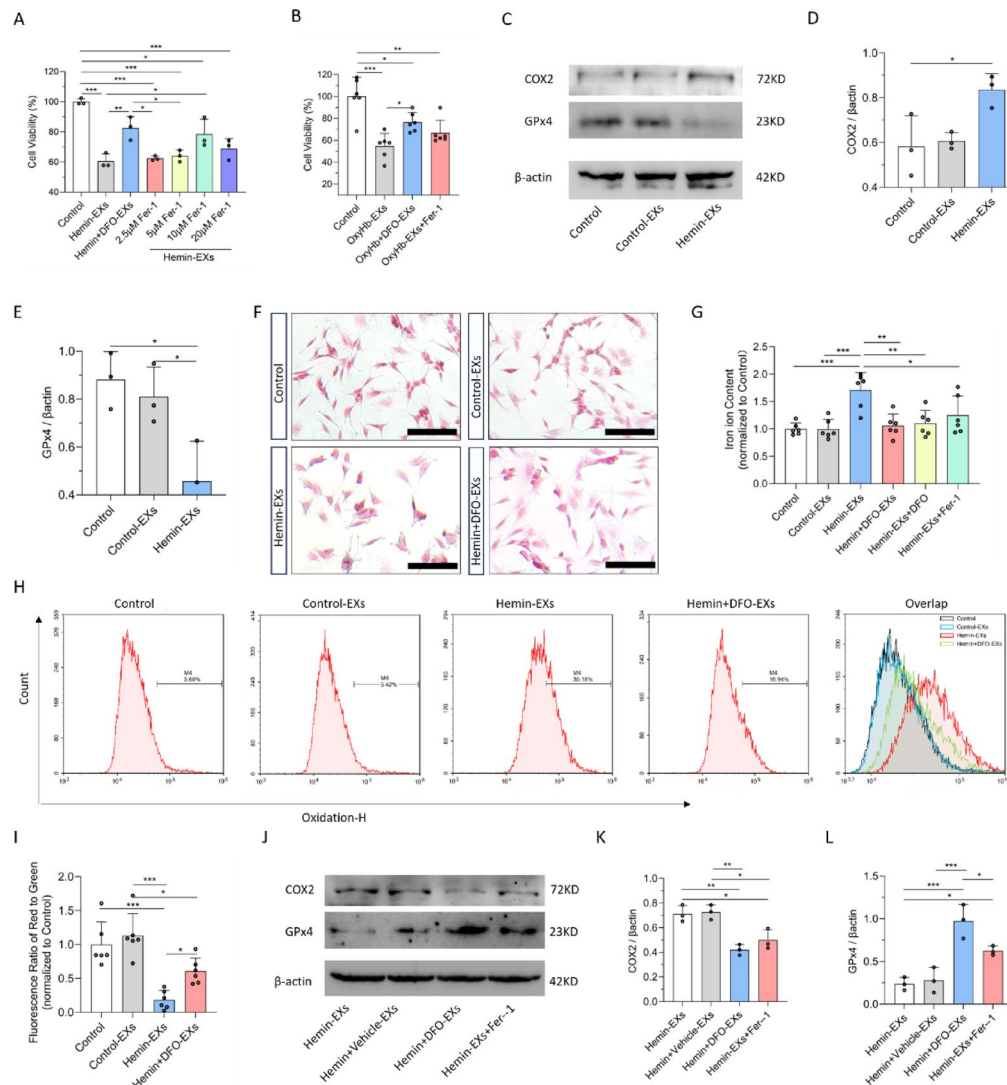


Fig. 5 Hemin-EX-induced neuronal ferroptosis by transducing iron ions. The CCK8 method showed both DFO and Fer-1 can improve the neuronal impairment induced by Hemin-EXs (**A**) and OxyHb-EXs (**B**), $n = 3$; (**C**) typical bands of COX2 and GPx4 indicate that Hemin-EXs induce the onset of ferroptosis in neurons; the statistical results show that COX2 (**D**) and GPx4 (**E**) increase significantly after MC-EX treatment, $n = 3$, bar = 50 μ m; (**F**) representative images of neurons stained with iron after treatment with EXs in each group; (**G**) analysis of iron-positive areas shows that Hemin-EXs induced an increase in neuronal iron levels by transducing the carried iron ions, $n = 6$; (**H**) representative results of flow cytometry analysis of lipid peroxide levels in neurons; (**I**) statistical analysis shows that excess iron ions carried by Hemin-EXs can cause lipid peroxidation damage in neurons, $n = 6$; (**J**) bands indicate the effects of iron ions in Hemin-EXs on COX2 and GPx4 expression in neurons; both DFO and Fer-1 inhibited the overexpression of the ferroptosis markers COX2 (**K**) and GPx4 (**L**), $n = 3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Statistical method: mean $\pm$ standard deviation (SD), ABDEGIKL: A one-way analysis of variance (ANOVA)

GPx4 ($P < 0.05$, Figs. 5J–L). In the iron-depleted MC-EXs (Hemin + DFO-EXs) group, there was also no significant overexpression of COX2 ($P < 0.01$ vs. Hemin-EXs) or downregulation of GPx4 ($P < 0.05$ vs. Hemin-EXs).

In animal studies, we first administered PKH67-labeled EXs intranasally to mice and assessed their uptake by NeuN-labeled neurons. Fluorescence analysis revealed that cortical neurons successfully internalized EXs, with fluorescence intensity at 6 h significantly higher than at 24 h ($P < 0.05$; Fig. 6A, B). The SAH mouse model was established (Fig. 6C), and assessments were performed

following three consecutive days of EXs administration. We observed that Hemin-EXs further enhanced the SAH-induced overexpression of COX2 ($P < 0.05$) and downregulation of GPx4 ($P < 0.05$; Fig. 6D–F). Furthermore, TEM observation revealed that Hemin-EXs significantly exacerbated SAH-induced mitochondrial damage, ultimately culminating in ferroptosis (Fig. 6G). Iron staining and MDA assays showed that Hemin-EXs significantly increased iron deposition in the cortical region ($P < 0.05$; Fig. 6H, I) and elevated MDA levels in brain tissue ($P < 0.05$; Fig. 6H–J). Interestingly, after

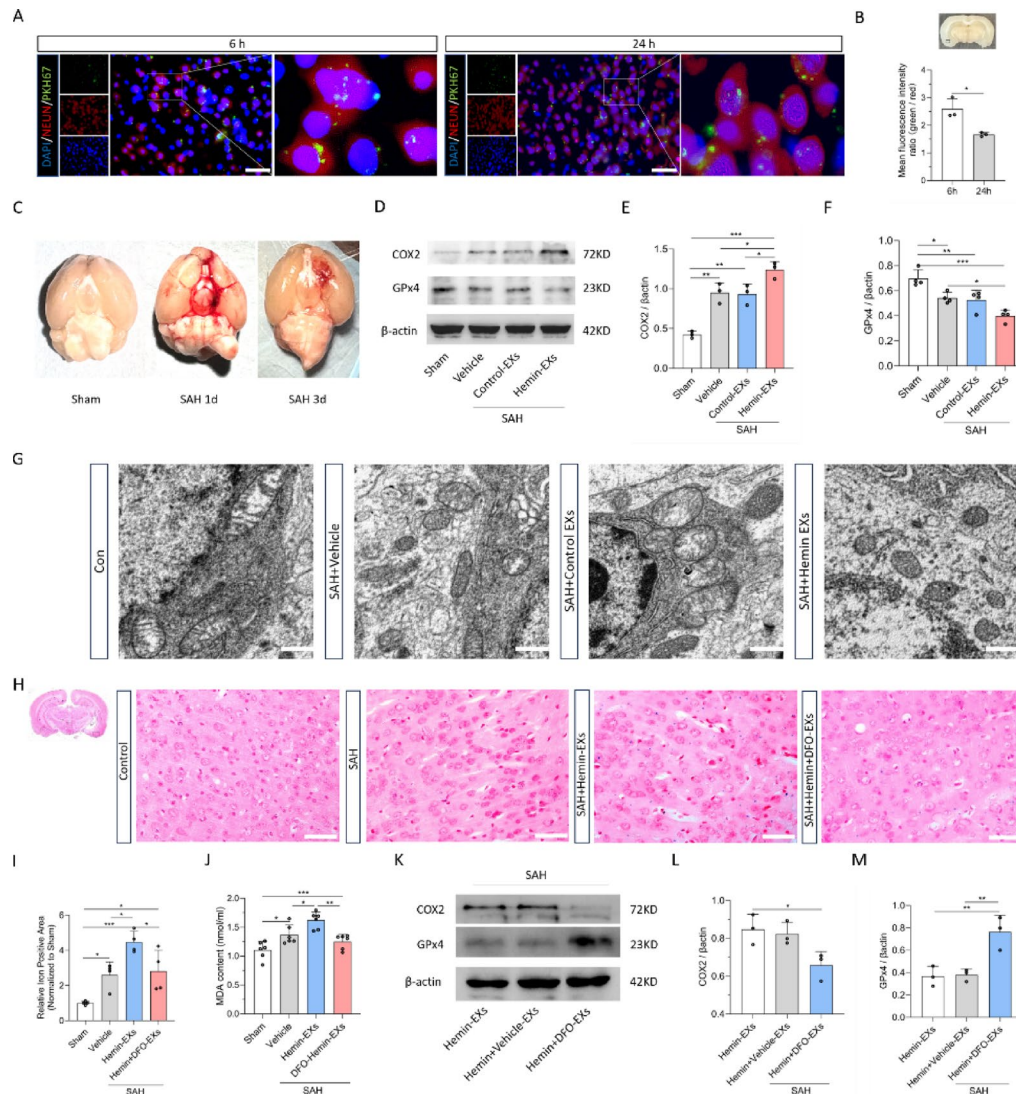


Fig. 6 Hemin-EXs aggravated iron-dependent ferroptosis damage in brain tissue of SAH mice. **(A)** representative images of EXs in neurons in brain tissue slices at different time points after nasal administration of PKH67-labeled MC-EXs, bar = 50 μm; **(B)** statistical graph of EXs fluorescence intensity in neurons of brain tissue at 6 h and 24 h, $n = 3$; **(C)** representative images of the brain tissue from mice in the sham group and SAH group; **(D)** bands represent the ferroptosis key proteins GPx4 and COX2 in brain tissues of mice from different groups; the statistical chart shows that Hemin-EXs aggravate the abnormal expression of COX2 **(E)** and GPx4 **(F)** after SAH, $n = 3$; **(G)** representative images of TEM of brain tissue sections, bar = 500 nm; **(H)** representative images of iron staining of brain tissue sections, bar = 50 μm; **(I)** quantitation of iron staining positive area in brain tissue sections, $n = 4$; **(J)** quantitation of MDA content in the brain tissue, $n = 6$; **(K)** representative bands showing the effects of iron-depleted EXs on the expression levels of GPx4 and COX2 in the brain tissue of SAH mice; **(L)** the statistical results of COX2 expression, $n = 3$; **(M)** the statistical results of GPx4 expression, $n = 3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Statistical method: mean $\pm$ standard deviation (SD), B:unpaired t test, I:paired t test, EFHKL: A one-way analysis of variance (ANOVA)

removing excess iron ions from EXs using DFO, the MC-EXs-induced iron accumulation ($P < 0.05$) and MDA elevation ($P < 0.01$) in brain tissue were markedly attenuated, and the aberrant expression of COX2 ($P < 0.05$ vs. Hemin-EXs) and GPx4 ($P < 0.01$ vs. Hemin-EXs) was significantly alleviated in the iron-depleted MC-EXs (Hemin + DFO-EXs) group (Fig. 6K–M). These results suggest that Hemin-EXs induced neuronal ferroptosis by transducing excess iron ions.

MC-EXs induce iron-dependent neurological impairment in mice after SAH

As shown in Figure S4A, we evaluated sensorimotor function, cognition, emotion, and muscle strength in each experimental group. The time points of the functional tests are indicated in Fig. 7A. In the RT, both the time on the rod and rod speed were reduced in the SAH group from days 1 to 14, and administration of Hemin-EXs further exacerbated these impairments between days 3 and 21 (Fig. 7B, C). In the ART, both the contact time and removal time were significantly prolonged in SAH mice

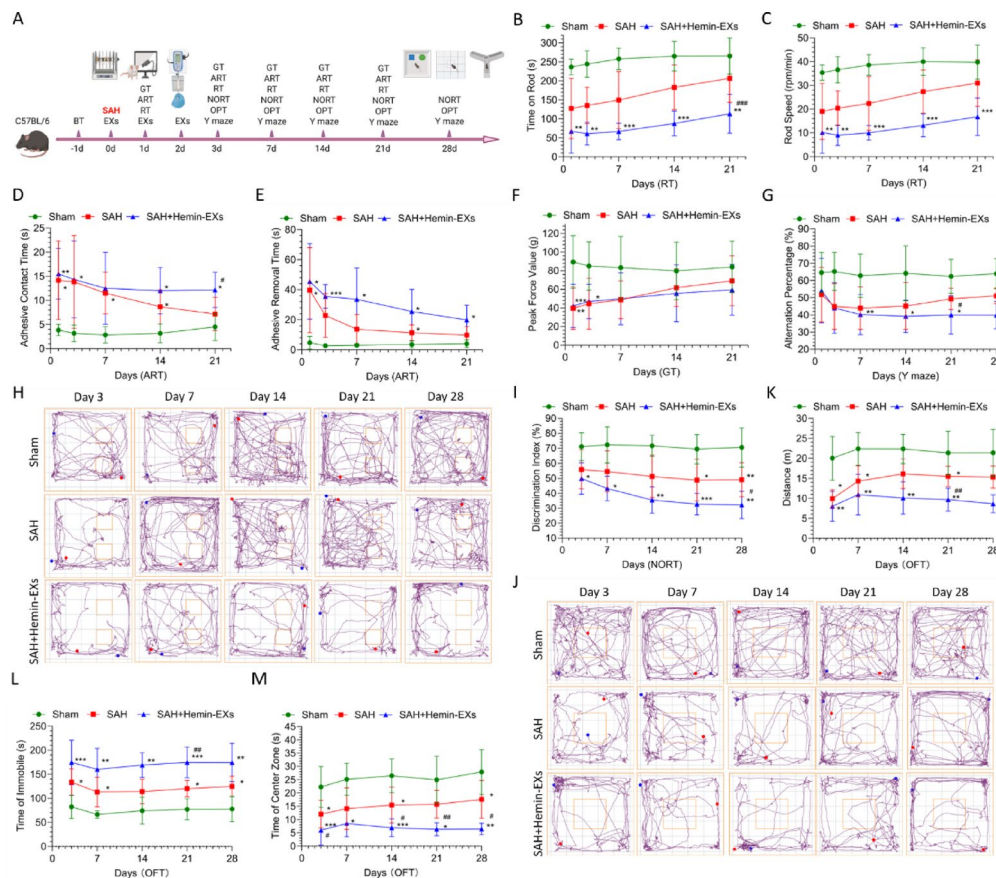


Fig. 7 Hemin-EXs aggravated motor and cognitive dysfunction in SAH mice. **(A)** Experimental timeline for EX administration and various neurological function assessments; rotarod test showed the Hemin-EXs the time on rod **(B)** and the rod speed **(C)** in SAH mice; adhesion removal assay confirmed that Hemin-EXs prolonged the time it took SAH mice to detect **(D)** and remove adhesions **(E)**; **(F)** the grip Test showed that MC-EXs had no significant effect on SAH mice; **(G)** MC-EXs aggravated alternations in the Y Maze after SAH; representative results **(H)** and statistics **(I)** of novel object recognition test showed that Hemin-EXs further reduced the discrimination index after SAH; in the open field test **(J)**, MC-EXs had a deteriorating effect on the movement distance **(K)**, center zone residence time **(L)**, and immobility time **(M)** of SAH mice. $n = 10$, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Statistical method: BCDEFGIKLM: two-way repeated-measures ANOVA

compared to the Sham group from days 1 to 3. Hemin-EXs further prolonged these times at day 21 ($P < 0.05$, Fig. 7D, E). In the GT, the peak forelimb force of both the SAH and SAH+Hemin-EXs groups declined from days 1 to 3, with no significant difference between the two groups (Fig. 7F). In the Y-maze test, the alternation rate in SAH mice decreased from days 7 to 28 compared to the Sham group ($P < 0.05$), and Hemin-EXs further reduced the alternation rate at day 21 ($P < 0.05$, Fig. 7G). In the NORT, the discrimination index in the SAH mice was significantly reduced from days 7 to 28 compared to the Sham group ($P < 0.05$). Hemin-EXs further impaired recognition memory from at day 28 ($P < 0.05$, Fig. 7H, I). In the OFT, the movement distance and center zone time were significantly reduced in SAH mice from days 3 to 28, while immobility time was increased, and Hemin-EXs administration further impaired these behavioral indicators from days 14 to 28 (Fig. 7J–M). To explore the role of iron ions, we treated Hemin-EXs with DFO to chelate

excess iron. Compared to the Hemin-EXs group, DFO-treated Hemin-EXs improved both time on the rod and rod speed in the RT from days 7 to 21 (Fig. 8A, B), but no improvement was observed in the GT (Figure S4B). DFO treatment also increased the alternation rate in the Y-maze from days 21 to 28 (Fig. 8C), shortened the contact and removal times of ART on day 21 (Figure S4C, Fig. 8D), improved the discrimination index in the NORT from days 14 to 28 (Fig. 8E, G), and recovered movement distance, center zone time, and immobility time in the OFT from days 14 to 21 (Fig. 8F, H–J). Collectively, these results suggest that Hemin-EXs aggravate impairments in sensorimotor, cognitive, and emotional functions after SAH, primarily through the transduction of excess iron ions.

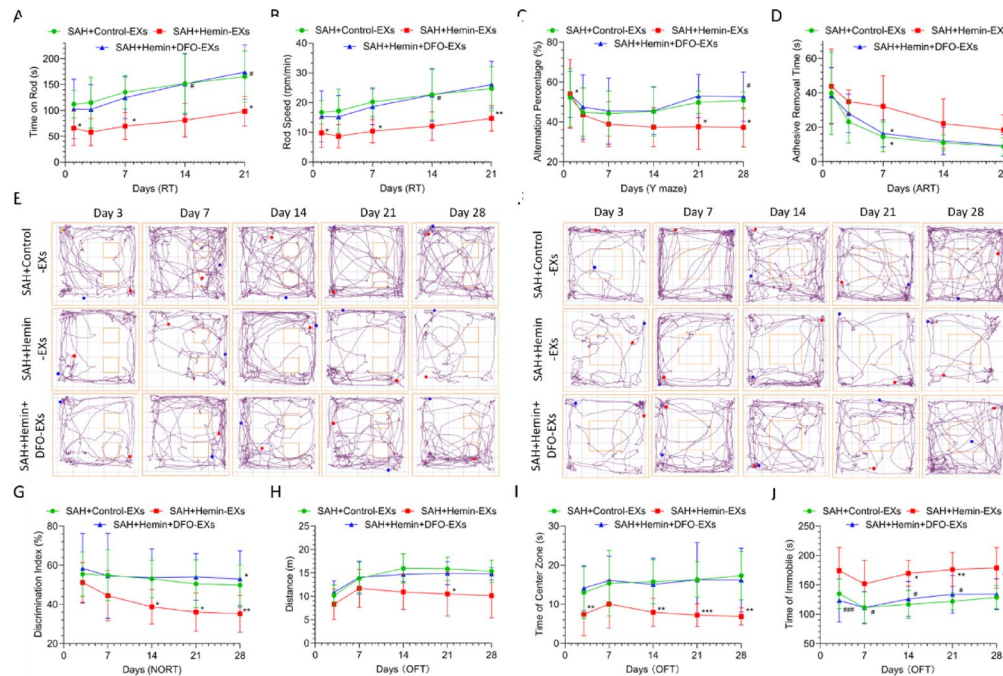


Fig. 8 Removal of iron ions inhibited the effects of Hemin-EXs on neurological function in SAH mice. Compared to Hemin-EXs, Hemin+DFO-EXs decreased the time on the rod (A) and the rod speed (B); alternations in the Y Maze (C) and adhesion removal time (D) were also improved in Hemin+DFO-EXs; representative results from the novel object recognition test (E) and open field test (F); statistical results show that using DFO to remove iron ions from Hemin-EXs reduces the effect on the discrimination index (G), movement distance (H), center zone residence time (I) and immobility time (J). $n = 10/12$ conditioned, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Statistical method: ABCD, GHJ: two-way repeated-measures ANOVA

C3/C5/NF- κ B pathway is involved in neuronal damage induced by iron-overloaded MC-EXs

Next, we investigated the molecular mechanisms underlying neuronal damage. By integrating the abovementioned transcriptomic data with the ferroptosis-related dataset GSE197104, we performed an intersection analysis (Fig. 9A) and identified six key genes—two down-regulated and four upregulated (Fig. 9B). The enrichment analysis suggested that NF- κ B serves as a critical downstream effector (Fig. 9C). Protein–protein interaction (PPI) analysis using the STRING database predicted that the C3/C5/NF- κ B axis was a potential signaling pathway involved in neuronal injury (Fig. 9D). GSEA further confirmed significant enrichment of the NF- κ B pathway in neurons treated with SAH-induced MC-EXs (NES = 1.66, $P < 0.001$; Fig. 9E). In vivo, we observed elevated expression levels of C3, C5, and NF- κ B in brain tissue following SAH ($P < 0.05$), and this abnormal upregulation was further exacerbated by administration of Hemin-EXs ($P < 0.05$ for C3 and C5; $P < 0.01$ for NF- κ B; Fig. 9F–I). In vitro experiments confirmed that Hemin-EXs significantly increased the expression of C3 ($P < 0.001$), C5 ($P < 0.01$), and NF- κ B ($P < 0.01$) in cultured neurons. However, treatment with Hemin+DFO-EXs attenuated the overexpression of these markers ($P < 0.05$; Fig. 9J–M). Collectively, these findings indicate that iron-overloaded

MC-EXs contribute to neuronal injury by activating the C3/C5/NF- κ B signaling pathway.

Inhibition of the C3/C5/NF- κ B pathway improves neuronal ferroptosis caused by MC-EXs after SAH

To verify the regulatory role of the C3/C5/NF- κ B pathway in Hemin-EXs-induced neuronal ferroptosis, we transfected neurons with C3 siRNA as shown in Fig. 10A. The results show that 4 μ L of C3 siRNA significantly reduced C3 expression levels in neurons ($P < 0.05$; Fig. 10B, C), and mitigated Hemin-EXs-induced neuronal viability loss ($P < 0.05$; Fig. 10D). Furthermore, C3 knockdown alleviated lipid peroxidation accumulation in neurons following Hemin-EXs exposure ($P < 0.05$; Fig. 10E, F) and partially restored the upregulation of COX2 ($P < 0.05$) and the downregulation of GPx4 ($P < 0.05$) (Fig. 10G–I). At the molecular level, we further confirmed that C3 siRNA transfection not only inhibited Hemin-EXs-induced overexpression of C3 ($P < 0.01$) but also suppressed the upregulation of C5 ($P < 0.05$) and NF- κ B ($P < 0.05$) in neurons (Fig. 10J–M). Additionally, molecular docking analysis revealed multiple potential binding domains between C5 and NF- κ B (Figure S5A). Collectively, these findings suggest that the C3/C5/NF- κ B signaling axis plays a key role in mediating Hemin-EXs-induced ferroptotic damage in neurons.

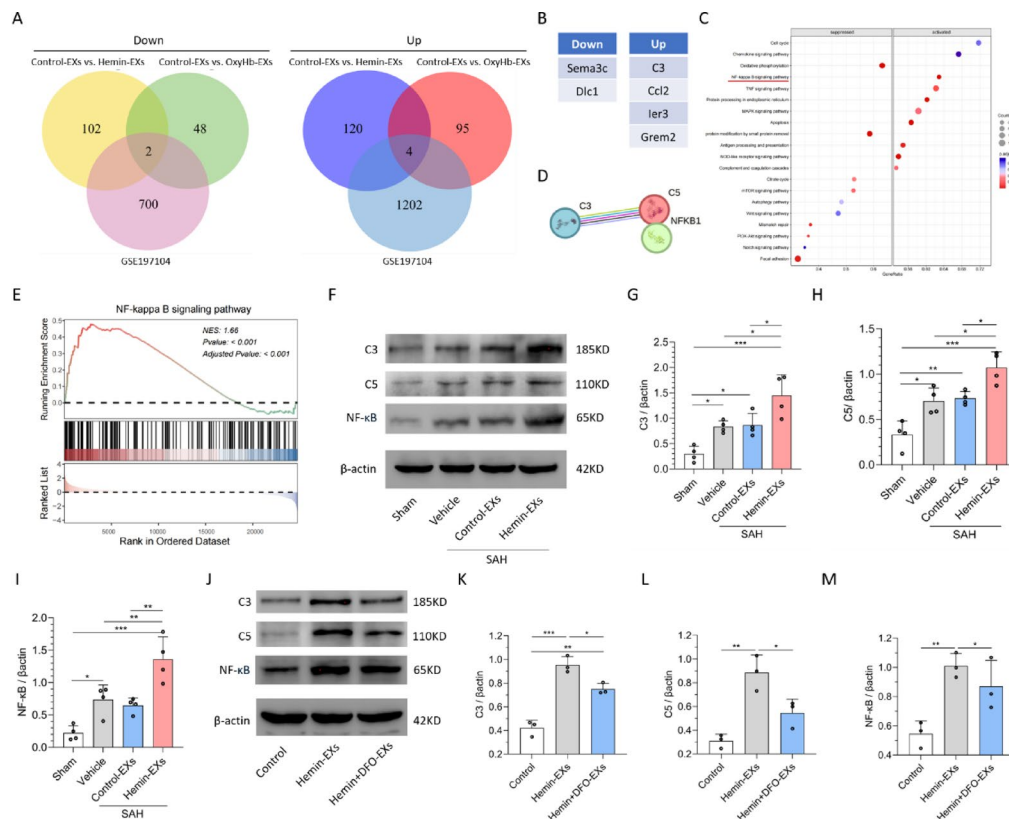


Fig. 9 Bioinformatics analysis and SAH model experiments predicted that the C3/C5/NF-κB pathway may be involved in Hemin-EXs-induced neuronal injury. **(A)** The upregulated and downregulated genes in our transcriptomics data were overlapped with the GSE197104 dataset to identify ferroptosis-related genes; **(B)** the list of screened genes; **(C)** KEGG pathway analysis of GSE184917 dataset revealed the pathways activated and suppressed following C3a treatment, and the NF-κB pathway showed the most significant impact; **(D)** the STRING database showed the interaction between C3, C5 and NF-κB; **(E)** GSEA analysis of our dataset revealed that genes related to the NF-κB pathway were significantly enriched; in SAH mice **(F)**, Hemin-EXs induced the overexpression of C3 **(G)**, C5 **(H)** and NF-κB **(I)**; in neurons in vitro **(J)**, $n=4$; removing iron ions with DFO decreased the Hemin-EXs induced overexpression of C3 **(K)**, C5 **(L)** and NF-κB **(M)**, $n=3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Statistical method: mean $\pm$ standard deviation (SD), GHIKLM: A one-way analysis of variance (ANOVA)

Discussion

This study aims to elucidate the novel mechanism underlying EX-mediated intercellular communication involved in iron-dependent neuronal injury and neurological dysfunction following hemorrhagic stroke. The principal findings are as follows: (1) Screening and confirmation that the iron content of MC-EXs is elevated in the SAH in vitro models and that they induce neuronal damage; (2) MC-EXs were found to be taken up by neurons via lipid raft-mediated, dynamin-dependent, clathrin-mediated, and caveolae-mediated endocytosis; (3) Bioinformatic prediction and experimental validation confirmed that iron-overloaded MC-EXs contribute to neuronal ferroptotic injury by delivering iron ions; (4) These EXs also exacerbated iron accumulation in the CNS of SAH mice, thereby aggravating sensorimotor, cognitive, and emotional deficits; (5) Finally, both prediction and verification indicated that the C3/C5/NF-κB signaling pathway mediates the neuronal response to MC-EX-induced injury. A

graphical summary of these key findings is presented in Fig. 1A.

After SAH, extracellular hemoglobin released from lysed erythrocytes triggers a cascade of toxic events. Hemoglobin is scavenged by haptoglobin and cleared via the CD163 pathway, while excess heme is bound by hemopexin and taken up through CD91. Both pathways release iron, which, if unregulated, contributes to oxidative stress, lipid peroxidation, ferroptosis, and inflammation [18]. Elevated levels of iron and lipid peroxidation products have been detected in the CSF of patients with SAH [19]. In postmortem brain tissue from patients with SAH, iron deposition exhibited a pattern of being more concentrated near the brain surface, and diminished with distance. Iron was found both inside cells, primarily in macrophages, and in extracellular spaces [20]. Nevertheless, iron deposition-related cellular injury persisted in brain regions distant from the hematoma core. This raises the question of whether EXs contribute to the long-range transport of iron ions and, if so, which specific cell types

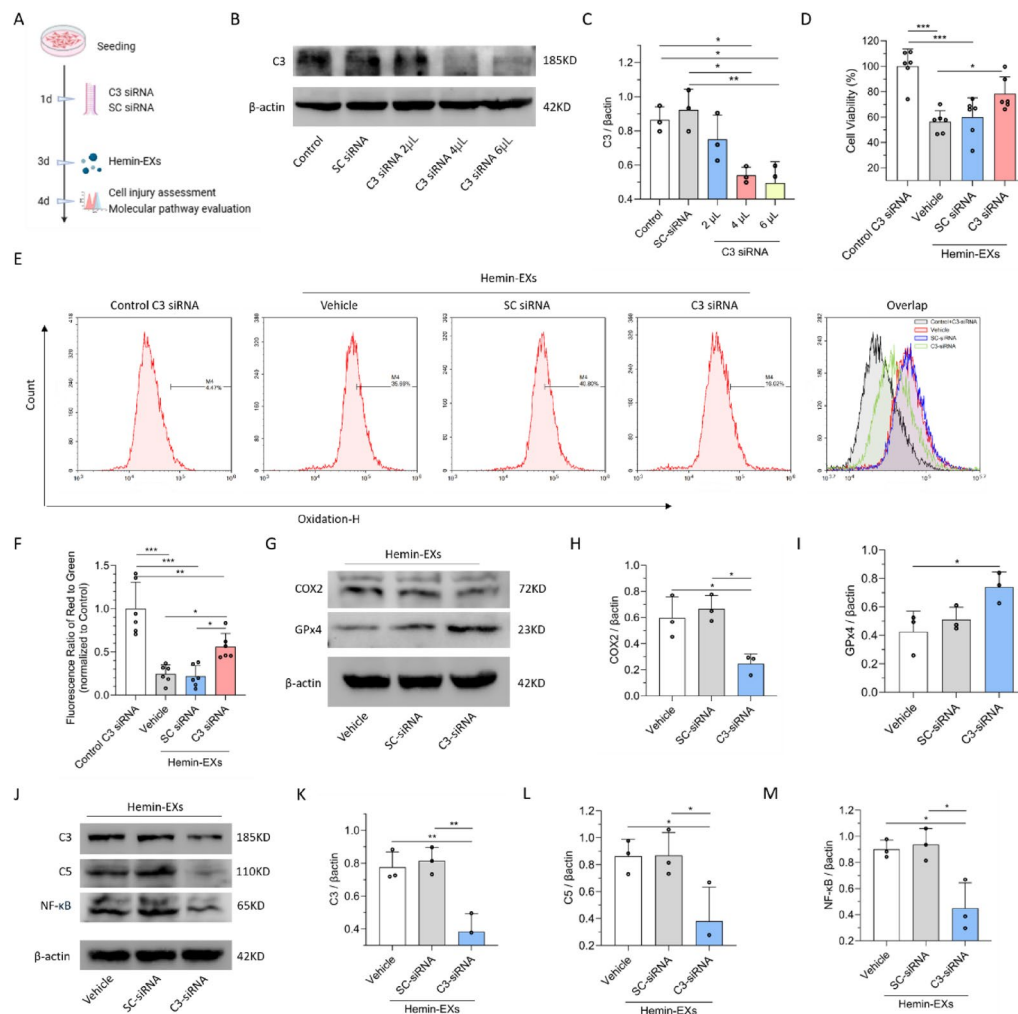


Fig. 10 Further verification of the involvement of the C3/C5/NF-κB pathway in Hemin-EXs-induced neuronal ferroptosis. **(A)** Experimental timeline for C3 knock down in neurons and following tests; **(B)** representative bands of C3 protein expression in neurons after different doses of C3 siRNA; **(C)** the analysis showed that 4 μL siRNA could significantly downregulate the expression of C3, $n=3$; **(D)** C3 siRNA improved the effect of Hemin-EXs on neuronal activity, $n=6$; **(E)** representative images of flow cytometry of lipid peroxidation staining in neurons; **(F)** the statistical graph showed that the lipid peroxidation level decreased in the C3 siRNA group, $n=6$; in the western blot results **(G)**, downregulation of C3 also inhibited the abnormal expression of Hemin-EXs-induced ferroptosis markers COX2 **(H)** and GPx4 **(I)**, $n=3$; in further detection of pathway proteins **(J)**, C3 siRNA also inhibited the upregulation of **(K)**, C5 **(L)** and NF-κB **(M)** in neurons induced by MC-EXs, $n=3$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Statistical method: mean $\pm$ standard deviation (SD), F:paired t test, CDH1KLM: A one-way analysis of variance (ANOVA)

are responsible for releasing EXs that mediate iron overload and subsequent neuronal damage. A study on HIV-1 Tat-stimulated astrocyte-derived EVs showed that they carried toxic amyloids to induce neuronal synaptodegeneration and Alzheimer's-like pathology, which could be mitigated by silencing astrocytic HIF-1 α [21]. This study demonstrated that EXs released by MCs in various in vitro SAH models contain significantly elevated iron levels. When co-cultured with neurons, these MC-EXs lead to increased intracellular iron accumulation and reduced neuronal viability. To further clarify the link between iron overload and neuronal injury, we treated MC-EXs with the iron chelator DFO, which significantly attenuated their neurotoxic effects. While previous studies

have explored the use of iron chelators in SAH models to alleviate iron-induced oxidative stress [22], our findings provide novel evidence for a specific EX-mediated mechanism underlying dysregulated iron metabolism and CNS injury.

Subsequently, we explored the pathways involved in the neuronal uptake of MC-EXs and characterized the resulting patterns of neuronal damage. Based on previous studies, we selected five primary mechanisms of cellular uptake of EVs: macropinocytosis, lipid raft-mediated endocytosis, dynamin-dependent endocytosis, clathrin-mediated endocytosis, and caveolae-mediated endocytosis [13, 23]. We then used specific molecular inhibitors to probe each pathway. This investigation identified four

main mechanisms for neuronal uptake of MC-EXs: lipid raft-mediated, dynamin-dependent, clathrin-mediated, and caveolae-mediated endocytosis. These pathways may serve as potential targets for future therapeutic intervention or regulatory strategies. Understanding the type of damage caused by neuronal uptake of MC-EXs became our next question. Our transcriptomic and pathway enrichment analysis of the affected neurons pointed to ferroptosis as a key injury mechanism. Ferroptosis is an iron-dependent form of regulated cell death, triggered by abnormal iron metabolism and extensive lipid peroxidation, ultimately leading to oxidative stress and neuronal death [24]. Consistent with this, we also observed signs of iron overload in neurons, prompting us to further investigate the involvement of ferroptosis. In Parkinson's disease research, it has been demonstrated that microglial CR3 promotes neuronal ferroptosis by driving NADPH oxidase 2 (NOX2)-mediated iron accumulation [25]. Moreover, iron dyshomeostasis and glial cell-mediated ferroptosis have been implicated in dopaminergic neuron loss and α -synuclein aggregation [26]. By applying the specific ferroptosis inhibitor and assessing hallmark features of ferroptosis both in vivo and in vitro, we confirmed that ferroptosis is indeed involved in MC-EXs-induced neuronal injury. This damage is primarily dependent on the transfer of excessive iron ions into neurons. Our findings reveal a novel mechanism of EXs-mediated ferroptotic injury following stroke.

Hemorrhagic stroke has been shown to cause long-term impairments in sensory, motor, emotional, and cognitive functions [27, 28], however, the specific mechanism that causes neurological damage remains unclear. In conditions such as aging and Parkinson's disease, elevated iron levels or dysregulated iron metabolism in the CNS have been directly linked to neurological deficits [5, 29]. Cerebral infarction has been linked to both brain iron overload and iron deficiency. Iron contributes to neuronal damage after stroke through oxidative stress, excitotoxicity, inflammation, and ferroptosis. Conversely, iron deficiency anemia is also a stroke risk factor. These mechanisms highlight the complex role of iron in stroke pathology [30]. Previous studies, including our own, have demonstrated that SAH is associated with iron accumulation in the cortex and can induce ferroptosis, leading to neurological dysfunction in experimental animals. Inhibiting ferroptosis has been shown to alleviate this damage [31, 32]. In the present study, we aimed to investigate whether neuronal iron overload induced by MC-EXs could exacerbate neurological injury following SAH. Through a number of behavioral tests on mice, we found that MC-EXs indeed aggravated mid- and long-term sensory, motor, emotional, and cognitive dysfunction, although limb muscle strength remained unaffected. Notably, iron ion removal from MC-EXs using DFO

significantly alleviated these functional impairments. Our findings suggest that iron-overloaded MC-EXs after SAH can exacerbate long-term neurological damage by promoting neuronal iron deposition and potentially triggering ferroptosis.

The connection between exosomal iron and C3 upregulation likely involves, at least in part, a direct transcriptional mechanism. This concept is supported by prior evidence indicating that intracellular iron can drive C3 expression via a non-canonical TGF- β pathway, independently of oxidative stress [33]. The clinical correlation between systemic iron/ferritin and C3 levels further underscores the potential pathophysiological relevance of this link [34]. We thus propose a plausible mechanism whereby iron-loaded MC-EXs activate this direct transcriptional pathway in neurons, thereby enabling C3 to function as an upstream initiator of the observed downstream C5/NF- κ B signaling and ferroptosis. We next sought to elucidate the molecular mechanism linking iron transduction by MC-EXs to neuronal ferroptosis. To this end, we performed bioinformatics and protein interaction analyses on our transcriptomic data. Our findings suggest that the upregulation of the C3/C5/NF- κ B signaling pathway may represent a potential underlying mechanism. In neuropsychiatric disorders, neuronal complement C3 has been shown to rescue synaptic and learning deficits, thereby mitigating cognitive dysfunction [35]. As a central factor of the complement system, C3 plays a pivotal role in the cascade. Upon activation, C3 is cleaved into C3a and C3b — a critical step in the complement response. C3b subsequently contributes to the formation of the C5 convertase, which lyses C5 into C5a and C5b [36]. C5a has been shown to activate downstream signaling pathways, including NF- κ B, through its receptors C5aR1 and C5aR2, under various pathological conditions [37, 38]. This study also confirmed in an SAH model that the expression of complement C3, C5, and NF- κ B in neurons was upregulated after SAH. Furthermore, iron-overloaded MC-EXs not only exacerbated this overexpression in affected neurons but also aberrantly activated the C3/C5/NF- κ B pathway in healthy neurons in vitro. These findings suggest that the proposed mechanism is indeed involved in MC-EX-induced neuronal injury. Consequently, investigating how this pathway regulates ferroptosis has become the next critical question. In other organ systems, a limited number of studies have implicated the complement C3 and C5 in the regulation of ferroptosis, so the underlying mechanisms remain largely unclear [39, 40]. The role of NF- κ B in ferroptosis appears to vary significantly across different disease contexts. For instance, in liver tumors, loss of leukaemia inhibitory factor receptor (LIFR) activates NF- κ B signaling via src homology 2 domain-containing phosphatase 1 (SHP1), leading to upregulation of the

iron-chelating cytokine lipocalin 2 (LCN2), which results in iron depletion and resistance to ferroptosis-inducing agents [41]. In spermatocytes, miR-342-5p targets ELKS/RAB6-interacting/CAST family member 1 (Erc1) to activate the NF- κ B pathway, which is essential for zinc oxide nanoparticle-induced ferroptosis [42]. In the present study, we investigated the regulatory role of the C3/C5/NF- κ B signaling axis in neuronal ferroptosis. We found that treatment with MC-EXs led to both increased neuronal ferroptosis and upregulation of the C3/C5/NF- κ B pathway, with a positive correlation between the two. Furthermore, silencing C3 expression using siRNA inhibited the activation of the C3/C5/NF- κ B pathway and attenuated MC-EXs-induced neuronal ferroptosis. These findings suggest that the C3/C5/NF- κ B pathway plays a critical role in mediating neuronal ferroptosis induced by iron-overloaded MC-EXs. While our loss-of-function data position C3 as a critical upstream regulator in this pathway, future studies employing more specific inhibitors or genetic models for C5 and NF- κ B will be valuable to fully dissect the precise contributions of each component.

In conclusion, our series of studies demonstrated that following SAH, EXs released by MCs carried excess iron ions and contributed to neuronal injury. After being taken up by neurons mainly through lipid raft-mediated, dynamin-dependent, clathrin-mediated, and caveolae-mediated endocytosis, MC-EXs transduced the excess iron ions they carry, leading to neuronal iron deposition, upregulation of the C3/C5/NF- κ B pathway, and thus causing ferroptosis and aggravating motor, sensory, and cognitive impairment after SAH.

Furthermore, while our study identified several endocytic pathways responsible for the uptake of MC-EXs by neurons, a functional link between specific blockade of these pathways and the attenuation of ferroptosis was not established here. Future studies employing genetic or pharmacological tools to selectively inhibit key uptake pathways (e.g., dynamin-dependent endocytosis) *in vivo* will be crucial to definitively confirm that neuronal internalization of iron-overloaded MC-EXs is a requisite step in the pathological cascade leading to ferroptosis and neurological deficits after SAH.

This study has certain limitations. For instance, only four types of neural cells were examined during screening; therefore, the potential effects of EXs derived from other cell types on neuronal function remain unclear. Additionally, the specific mechanisms underlying the upregulation of complement C3 following iron deposition in neurons warrant further investigation. However, our findings provide compelling evidence for the role of EXs in the pathophysiological progression of SAH. Moreover, this study offers valuable insights into potential

molecular mechanisms and highlights new directions for future therapeutic research in SAH.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12951-025-03974-y>.

Supplementary Material 1

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Author contributions

Y. L. and B. S. conducted the experiments, analyzed the data, and wrote the manuscript. Z. Y. and X. L. provided technical support and assisted in data collection. H. S. contributed to immunohistology experiments and manuscript revision. L. H. and X.-A. W. participated in the interpretation of results and edition of the manuscript. P. W., F. M. and J. C. participated in the creation of the figures. H. S. and J. B. conceived and supervised the project, secured funding, and finalized the manuscript. All authors reviewed and approved the final version.

Data availability

No datasets were generated or analysed during the current study.

Declarations

Competing interests

The authors declare no competing interests.

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