Targeting Ferroptosis Promotes Functional Recovery Through Mitigating White Matter Injury Following Acute Carbon Monoxide Poisoning

ShuHong Wang  
Zunyi Medical College: Zunyi Medical University

Binyuan Xiong  
Zunyi Medical College: Zunyi Medical University

Yin Tian  
Zunyi Medical College: Zunyi Medical University

Quan Hu  
Zunyi Medical College: Zunyi Medical University

Xuheng Jiang  
Zunyi Medical College: Zunyi Medical University

Ji Zhang  
Zunyi Medical College: Zunyi Medical University

Lin Chen  
Zunyi Medical College: Zunyi Medical University

Ruilie Wang  
Zunyi Medical College: Zunyi Medical University

Mo Li  
Zunyi Medical College: Zunyi Medical University

Xin Zhou  
Zunyi Medical College: Zunyi Medical University

Tianxi Zhang  
Zunyi Medical College: Zunyi Medical University

Hongfei Ge (✉️ hongfei0723@163.com)  
Zunyi Medical College: Zunyi Medical University  
https://orcid.org/0000-0002-6232-2107

Anyong Yu  
Zunyi Medical College: Zunyi Medical University

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Abstract

Survivors suffering from acute carbon monoxide poisoning (ACMP) are apt to develop white matter injury (WMI). While, the mechanism that ACMP evokes WMI remains unclear. Given that ferroptosis plays an evident role in igniting oligodendrocyte damage to deteriorate WMI, exploring regimens to attenuate ferroptosis is a feasible approach to alleviate WMI post-ACMP. Here, the results indicated that ACMP induced WMI to evoke motor impairment resulting from the surplus iron and reactive oxygen species (ROS) accumulation after ACMP. And, the administration of ferrostatin-1 reduced iron and ROS deposition to repress ferroptosis, thereafter reducing WMI to promote motor recovery. Furthermore, the result demonstrated that the nuclear factor erythroid-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathway was involved in attenuating ferroptosis resulting from the application of ferrostatin-1. The present study offers a rationale that targeting ferroptosis to alleviate WMI is a feasible therapeutic strategy for ACMP.

1. Introduction

Acute carbon monoxide poisoning (ACMP) is one of the most common clinical emergencies with high mortality, and survivors are apt to develop a certain degree of brain injury after ACMP [1, 2]. Approximately 30–50% of survivors suffer from delayed encephalopathy following ACMP (DEACMP), also known as “lucid interval” ranging from 2 to 60 days with a mean duration of 22 days, that is characterized by a series of neuropsychiatric and neurological symptoms including coma, unresponsiveness, dementia, incontinence, psychiatric symptoms, low intelligence and increased muscle tension in the extremities [2–5]. To date, the pathogenesis of DEACMP is not fully understood. Previous studies have focused on exploring therapeutic strategies against ischemia, hypoxia, apoptosis, neurocytotoxicity, and neuroinflammatory damage of carbon monoxide [2, 6–9], but no rational explication has been identified [2]. Head magnetic resonance imaging (MRI) scans of patients with DEACMP show extensive white matter (WM) lesions appear at the periventricular, occipital, frontal, semioval centers, and the corpus callosum [10–12], indicating that white matter injury (WMI) plays a significant role in DEACMP progression. While, the mechanism that ACMP evokes WMI remains unclear.

Ferroptosis, a form of regulated cell death (RCD), is driven by the iron-dependent accumulation of excessive lipid peroxidation due to surplus deposition of reactive oxygen species (ROS) [13–15]. Previous studies have revealed that the oxidative stress derived from the accumulation of ROS, free radicals, and neuronal nitric oxide usually emerges plethoric lipid peroxidation following ACMP [16, 17]. Oligodendrocytes, which are mainly responsible for myelin formation of WM in the central nervous system (CNS) [18, 19], are rich in unsaturated fatty acid [4] and prone to ferroptosis-initiated CNS injury [20]. Furthermore, our previous study has demonstrated that ferroptosis plays an evident role in igniting oligodendrocyte damage to deteriorate WMI after spinal cord injury (SCI) [21]. Hence, exploring regimens to attenuate ferroptosis is a feasible approach to alleviate WMI post-ACMP.
Ferrostatin-1, an effective inhibitor of ferroptosis, might abate WMI although reducing ferroptosis following ACMP. Previous studies have shown that ferroptosis is a significant pathogenesis of deteriorating neurological deficits after CNS injury, and ferrostatin-1 exerts neuroprotective effects in various CNS diseases including cerebral ischemic stroke (CIS) [22, 23], intracerebral hemorrhage (ICH) [24–26], traumatic brain injury (TBI) [27, 28], subarachnoid hemorrhage (SAH) [29], and neurodegenerative diseases [30–32]. These evidence pinpoints the notion that ferrostatin-1 possesses the ability of penetrating the blood brain barrier (BBB) to exert neuroprotective effects. Most recently, our previous study corroborates ferrostatin-1, reduces iron and ROS accumulation, and downregulates the ferroptosis-related genes and its products of iron responsive element binding protein 2 (IREB2) and prostaglandin-endoperoxide synthase 2 (PTGS2) to further inhibit ferroptosis in oligodendrocytes, finally mitigates WMI to boost functional recovery post-SCI in rats [21]. However, the effect of ferrostatin-1 on functional recovery after ACMP remains unclear.

Here, we assumed that ferroptosis inflicted WMI due to iron and ROS overload, and ferrostatin-1 alleviated ferroptosis to assuage WMI following ACMP. To test our hypothesis, the mouse ACMP model was firstly established, and the CO concentration was determined. Then, the iron content and ROS level were assessed, and the optical density of WM was visualized using Luxol fast blue (LFB). Next, the effect of ferrostatin-1 on functional recovery was evaluated using behavioral tests including open field test (OFT) and beam walking test (BMT). Thereafter, the mechanism of ferrotatin-1 repressing ferroptosis was examined using immunofluorescence staining and western blot assays. The aim of the present study is to deepen the understanding of ferroptosis, and to expand the therapeutic effect of ferrostatin-1 on WMI post-ACMP, thereafter providing a therapeutic candidate for ACMP, even for other acute CNS diseases with the presence of ferroptosis.

2. Materials And Methods

2.1. Animals

A total of 257 adult C57BL/6 mice (male, 10–12 weeks, 22–26 g, 252 used for experiments and 5 died during experiments) were obtained from Animal Experimental Center of Zunyi Medical University. All procedures were approved by the local authorities of Zunyi Medical University for the laboratory use of animals (approve no. KLLY(A)-2021-033) and supervised by the Ethics Committee of Affiliated Hospital of Zunyi Medical University. All experimental procedures were performed according to the China’s animal welfare legislation for the protection of animals used for scientific purposes. Before and after treatment, all mice were given free access to food and water under the condition of constant temperature (22–25°C) moisture (55–60%), and photoperiod (12-h light/dark cycle). Every effort was made to reduce the number and sufferings of mice used for scientific purposes.

2.2. Mouse ACMP model

The model used in this study was performed as previously described [33]. Briefly, mice were placed in a temperature-controlled (22–24°C) transparent plastic chamber. Two carbon monoxide (CO) detectors
were installed at the center and margin of the chamber, respectively. Then, CO gas was filled into the chamber, and mice were firstly exposed to 1000 ppm CO in air for 40 min, followed by 3000 ppm CO for another 20 min until loss of consciousness. Subsequently, they were removed from the CO-rich environment and allowed them to breathe air until gain of consciousness.

2.3. Experimental groups and drug administration

After surgery, mice were randomly assigned into the following groups:

1) Sham group. Mice were placed in the same chamber for the same duration, but without CO gas treatment. Then, mice were intraperitoneally injected the same volume of 0.9% NaCl (containing 0.1% DMSO), equivalently to the volume of ferrostatin-1 in ACMP + ferrostatin-1 (ACMP + Fer-1) group, using the same method.

2) ACMP group. Mice were received CO gas administration. Afterward, mice were intraperitoneally administrated the same volume of 0.9% NaCl (containing 0.1% DMSO), equivalently to the volume of ferrostatin-1 in ACMP + Fer-1 group, using the same method.

3) ACMP + Fer-1 group. Ferrostatin-1 (Fer-1; Sigma-Aldrich, Munich, Germany) was administered intraperitoneally at a dose of 10 mg/kg body weight, as previously described [20]. Fer-1 was diluted in 0.9% NaCl after dissolved in DMSO.

2.4. Blood CO concentration measurement

Blood CO concentration was determined by a dual-wavelength CO quantitation kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer’s instructions. Briefly, 0.05 ml blood was collected from the heart and used for preparation of measurement samples (Measurement) and control specimens (Control). Then, the optical density value was determined at 568 and 581 nm using a micro-plate reader (Varioskan Flash, Thermo Fisher Scientific, Waltham, USA), respectively. ΔOD value was obtained using the following formula: ΔOD = Measurement ΔOD (A_{568} - A_{581}) - Control ΔOD (A_{568} - A_{581}). Afterward, the content of hemoglobin (Hb) was determined by the formula: Hb (g/l) = A_{540} × 73.54. Subsequently, the percentage of carboxyhemoglobin (COHb) was calculated using the regression equation following the formula: COHb (%) = (0.822 × ΔOD + 0.001) × 100%. Subsequently, the CO content was measured using the following formula: CO concentration = [COHb (%) × Hb (g/l) × 10^6 × 4]/64456.

2.5. Behavioral Tests

The open field test (OFT) was implemented to assess the locomotion recovery of mice on days 1, 3, 7, and 14 in each group, as previously described [34]. The field was equally divided into four adjacent but isolated chambers, of which were 50 cm long × 50 cm wide × 50 cm high. Each mouse was individually placed in the same chamber to move freely for 5 min and videotaped. Then, the chamber was cleaned up for the next test. Afterward, the videos were analyzed by two independent examiners, who were blinded to the experimental group, to measure the overall distance and average velocity of free movement using a software (ViewPoint Behavior Technology, Lyon, France).
The beam walking test (BWT) was performed to measure the coordination of mice after ACMP [35]. In brief, a 1-cm thick, 100-cm long wooden beam was installed at 50 cm above the ground. Before the CO administration, each mouse from each group was allowed to walk from one end of the beam to the other one for several times until they could fluently pass by themselves. After surgery, each mouse was allowed to walk from one side to the other side for 3 times during each time point and videos were recorded. Subsequently, the times of hindlimb flip from the beam (hindlimb fault) due to disability during 3 times traveling were calculated from the video by two independent examiners blinded to the experimental groups.

2.6. Luxol fast blue (LFB) staining

Mice were transcardially perfused with 0.1 M phosphate-buffered saline (PBS) after being anesthetized by 2% isoflurane/air mixture (2–3 l/min) on day 14. Brain tissues were dissected according to standard procedures and prepared for paraffin sectioning [36]. After being fixed with 4% paraformaldehyde (PFA), the specimens were dehydrated and cut into 5–8 um thick sections using a microtome (RM2235, Leika, Wetzlar, Frankfurt, Germany). The samples were dewaxed and put into solid blue staining solution overnight at room temperature. Afterward, they were washed with 95% ethanol to remove excess staining solution and rinsed with distilled water. Thereafter, the samples were underwent the firm-stained differentiation solution for 15 sec; 70% ethanol for color separation for 30 sec and washed with water. Subsequently, the samples were observed under a microscope until the white matter bundles were clearly defined and counterstained with tar violet staining solution for 30–40 sec followed by being washed with water. Thereafter, the specimens were mounted after dehydration with graded ethanol. Myelin sheaths were stained in blue and neurons were in pink or purple. Images were captured using a light microscope (Axio lab, Zeiss, Weimar, Germany) and analyzed by an Image J software (ImageJ 1.8, NIH, USA).

2.7. Perl's blue staining

Perl's blue staining was used to determine iron deposition using the 3-diaminobenzidine (DAB)-enhanced Perl's blue kit (Solarbio, Beijing, China) on day 14, as previously described [37]. Briefly, paraffin sections (3–7 µm thickness) of brain tissue were processed through a graded ethanol series, immersed in xylene, and then rehydrated in phosphate-buffered solution (PBS). Afterward, the sections were incubated in 10 µg/ml proteinase K solution containing 0.1% Triton X-100 for 20 min at room temperature. After being washed with PBS, the specimens were immersed in the Perl's blue solution (1% potassium ferrocyanide, 1% Triton X-100, 0.125 N HCl) for 30 minutes. The sections were washed three times with PBS, counterstained with dropwise counterstain solution for 3–5 min, rinsed with distilled water, dehydrated with gradient ethanol, and then mounted on glass slides with 10% neutral balsam. Images were photographed by a light microscope (Axio lab, Zeiss, Weimar, Germany) and examined by an Image J software (ImageJ 1.8, NIH, USA).

2.8. Iron concentration determination

Brain tissue samples were collected and homogenized under ice-cold conditions on days 1,3,7, and 14. Iron concentration was determined using a tissue iron assay kit (Nanjing Jiancheng Bioengineering
Institute, Nanjing, China) according to the manufacturer’s instructions. The optical density (OD) values were measured at 520 nm using a spectrophotometer (Varioskan Flash, Thermo Fisher Scientific, Waltham, MA, USA), and then the iron concentrations in all samples were determined by comparing the OD values of the samples to the standard curve.

2.9. Dihydroethidium (DHE) staining

DHE staining was applied to exhibit the levels of ROS. In short, brain sections were immersed in 10 µM Dihydroethidium (DHE) solution (MedChemExpress, Shanghai, China) for 1 h in the dark at 37°C. After being washed with PBS for 3 times, 4,6-diamidino-2-phenylindole (DAPI; Beyotime, Shanghai, China) was used to counterstain cell nuclei for 5 min at room temperature. Subsequently, the samples were mounted on neutral balata and observed with a fluorescent microscope (Carl Zeiss, Weimar, Germany). The positive area was calculated using an Image J software (ImageJ 1.8, NIH, USA).

2.10. Malondialdehyde (MDA) tests

MDA tests were conducted to measure the levels of lipid peroxidation by an MDA kit (R&D Systems, Minneapolis, MN, USA). Briefly, the fitted standard curve was established to determine the levels of MDA concentration. The brain samples were collected for lysis and centrifuged. The supernatants were firstly underwent acidification and then centrifuged for 3 times. Afterward, the samples were mixed with TBARS reagent for 2–3 h at 40–50°C. The OD value was determined at 532 nm using a spectrophotometer (Varioskan Flash, Thermo Scientific, Waltham, MA, USA), then the MDA concentration was calculated by fitting to the standard curve.

2.11. Immunofluorescent (IF) staining

For immunofluorescent staining, brain slices were incubated in 4% paraformaldehyde in 0.01 M PBS for 30 min at room temperature and permeabilized with 0.3% Triton-X 100 (Sigma-Aldrich, St. Louis, MO) in PBS. Then, samples were incubated with the following antibodies overnight at 4°C after blocking with 5% bovine serum album (BSA, Sigma-Aldrich, St. Louis, MO): anti-MBP antibody (cat. no. 10458-1-AP, Proteintech, Wuhan, China), anti-dMBP (cat. no. MBS618031, MybioSource, San Diego, CA, USA), anti-NF200 (cat. no. 60331-1-Ig, Proteintech, Wuhan, China), anti-GPX4 (cat. no. 67763-1-Ig, Proteintech, Wuhan, China), and anti-NRF2 (cat. no. 16396-1-AP, Proteintech, Wuhan, China). On the second day, the sections were incubated in Alexa Fluor 488- or 555-conjugated secondary antibodies (1:1000, Thermo Fisher Scientific, Waltham, MA, USA) for 2 hours at room temperature. Afterward, the cell nuclei were counterstained with DAPI for 10 min at room temperature. Finally, the specimens were mounted onto glass slides, and images were captured using a confocal microscope (Carl Zeiss, LSM780, Weimar, Germany) and examined by a Zen 2011 software (Carl Zeiss, Weimar, Germany).

2.12. Immunohistochemistry (IHC)

The brain samples were sliced into 4 µm thick sections using a microtome. Then, the slices were dewaxed and antigen- repaired. Afterward, the samples were incubated in hydrogen peroxide to remove endogenous
peroxidase for 10 min at room temperature. After being blocked with 5% bovine serum albumin (BSA), the specimens were incubated in primary antibodies of anti-HO-1 (cat. no. 10701-1-AP, Proteintech, Wuhan, China) and anti-FTH1 (cat. no. 10727-1-AP, Proteintech, Wuhan, China) overnight at 4°C. Afterward, the samples were immersed in secondary antibody with goat anti-rabbit immunoglobulin G (ZSGB-BIO, Beijing, China). Thereafter, DAB was applied to determine the positive cells in relative color. Images were photographed by a light microscope (Axio lab, Zeiss, Weimar, Germany) and analyzed by an Image J software (ImageJ 1.8, NIH, USA).

2.13. Western blot

The tissue lysates were collected, and the protein concentration of each sample was determined using the bicinchoninic acid (BCA) method by a BCA kit (Beyotime, Shanghai, China). Proteins (50 µg) from each sample were separated by 10% SDS-PAGE under reducing conditions and electro-blotted to polyvinylidene difluoride (PVDF, Roche, Indianapolis, IN, USA) membranes. Then, the membranes were blocked with 5% (w/v) non-fat dry milk (Beyotime, Shanghai, China) in tris buffered saline (TBS) with 0.1% Tween-20 (TBST) at room temperature for 2 h. Subsequently, the membranes were incubated with primary antibodies, anti-MBP antibody (cat. no. 10458-1-AP, Proteintech, Wuhan, China), anti-dMBP (cat. no. MBS618031, MybioSource, San Diego, CA, USA), anti-GPX4 (cat. no. 67763-1-Ig, Proteintech, Wuhan, China), anti-NRF2 (cat. no. 16396-1-AP, Proteintech, Wuhan, China), anti-HO-1 (cat. no. 10701-1-AP, Proteintech, Wuhan, China), anti-FTH1 (cat. no. 10727-1-AP, Proteintech, Wuhan, China), anti-IREB2 antibody (cat. no. 23829-1-AP, Wuhan Sanyiing,China), anti-COX2 (cat. no. A00048, Boster, Wuhan, China), anti-FSP1 (cat. no. 20886-1-AP, Proteintech, Wuhan, China), anti-β-Tubulin (cat. no. 10068-1-AP, Proteintech, Wuhan, China) or anti-GAPDH (cat. no. AF0006; Beyotime, Shanghai, China) overnight at 4°C. Afterward, the membrane was incubated with the corresponding horseradish peroxidase (HRP)-conjugated secondary antibody after being rinsed with TBST for three times. All membranes were visualized by a ChemiDoc™ XRS+ image system (Bio-Rad, California, USA) using a WesternBright ECL Kit (Advansta, Menlo Park, CA, USA). Densitometric measurement of each membrane was implemented using an Image Lab™ software (Bio-Rad, California, USA).

2.14. Transmission electron microscopy (TEM)

TEM was performed as previously reported [38]. Mice were anesthetized using isoflurane, and brain tissues of the basal ganglia were quickly removed after being flushed with 0.9% saline. Approximately 1 mm³ brain tissues were obtained and incubated in 1.25% glutaraldehyde overnight 4°C. After being post-fixed with 1.25% glutaraldehyde, the tissues were washed 3 times and fixed with 1% citric acid (OsO4) for 2 h. Then, gradient acetone was used for dehydration after being redyed with uranyl acetate. Thereafter, the samples were infiltrated with propylene oxide, embedded in epoxy, and cut into 70–90 nm slices using an ultramicrotome (EM UC7, Leica, IL, USA). Afterward, the samples were counterstained with lead citrate after placing on the copper trough grid, and the ultrastructure of mitochondria was observed using a transmission electron microscope (Hitachi HT7700, Tokyo, Japan). For each sample, at least three independent samples per group were used for analysis.
2.15. Statistical analysis

All data were presented as mean ± SD. The statistical analysis was performed using the SPSS 18.0 software (SPSS, Inc., Chicago, IL, United States). Comparisons between two groups were analyzed using Student’s t test or multiple t test if the data followed normal distribution by Shapiro-Wilk normality test. For comparisons among more than two groups, the data conforming to normal distribution were analyzed by one-way analysis of variance (ANOVA), followed by Tukey’s post hoc test. A $p < 0.05$ was considered statistical significance.

3. Results

3.1. ACMP caused motor deficits resulting from WMI.

To evaluate whether WMI occurred to induce functional impairment after ACMP, the mouse ACMP model was firstly established, and a series of measurements were performed (Fig. 1A). Firstly, the CO content illustrated that the CO concentration was significantly increased to the average level of 140 µmol/L in ACMP group, while it was at a super low level in sham group (Fig. 1B), indicating the mouse ACMP model was successfully constructed. Meanwhile, the behavioral tests including OFT and BMT showed that mice in ACMP group exhibited severe motor impairment (Fig. 1C, D). Then, the immunoblot bands depicted that the expression of MBP was markedly decreased in mice after ACMP (Fig. 1E, F). Subsequently, the LFB staining presented that the optical density of WM was obviously reduced in ACMP group (Fig. 1G, H). Coincidently, the immunostaining of NF200 and MBP was performed to assess the WMI in mice after ACMP. The representative images showcased that the optical density was profoundly decreased in ACMP group (Fig. 1I-K). Conversely, the expression of dMBP was evidently increased in ACMP group (Fig. 1L, M). Simultaneously, the optical density of dMBP was remarkably elevated in mice after ACMP (Fig. 1N, O).

Together, these results illustrated that mice receiving ACMP disclosed motor impairments due to WMI.

3.2. ACMP induced WMI due to ferroptosis.

Considering that WM is susceptible to oxidative stress [20], the iron and ROS accumulation were determined. Meanwhile, the ferroptosis was investigated using various measurements (Fig. 2A). The results indicated that the iron content was clearly increased from day 3 to 14 in ACMP group, compared with sham group (Fig. 2B). In the meantime, the Perl’s blue staining showed that the positive area was significantly increased after ACMP on day 14 (Fig. 2C, D). Then, the concentration of MDA was markedly increased (Fig. 2E), delineating that redundant lipid peroxidation occurred after ACMP. Afterward, the ROS level was determined using DHE staining. The results indicated that the ROS level was prominently elevated after ACMP (Fig. 2F, G), showcasing that ROS accumulation initiated redundant lipid peroxidation after ACMP. Collectively, these results illustrated that ACMP aroused excessive iron and ROS accumulation after ACMP. With respect to that ferroptosis is a form of RCD regulated by iron-dependent deposition of plethoric lipid peroxidation [13–15], the expression of ferroptosis-related protein of iron responsive element binding protein 2 (IREB2), an indicator of ferroptosis [21], was determined using
western blot assays. The bands depicted that the expression of IREB2 was substantially upregulated after ACMP (Fig. 2H, I). At the same time, the expression of cyclooxygenase 2 (COX2), another indicator of ferroptosis [39], was increased after ACMP (Fig. 2J, K). Afterward, the TEM images delineated that the fraction of shrunken mitochondria was markedly increased after ACMP (Fig. 2L, M). Collectively, these results showcased that ACMP evoked WMI due to ferroptosis.

### 3.3. Targeting ferroptosis with the administration of ferrostatin-1 improved motor deficits by reducing WMI after ACMP.

Given that ferrostatin-1 holds the ability of reducing WMI through suppressing ferroptosis [21], the effect of ferrostatin-1 application at a dosage of 10 mg/kg body weight, which is in line with previous report [20], on WMI after ACMP was investigated by varying approaches (Fig. 3A). Firstly, the behavioral tests including OFT and BMT showed that mice in ACMP + Fer-1 group exhibited better motor recovery than that in ACMP group (Fig. 3B, C). Then, the immunoblot bands delineated that the expression of MBP was evidently upregulated in ACMP + Fer-1 group than that in ACMP group (Fig. 3D, E). Coincidently, the LFB staining showed the optical density of WM in ACMP + Fer-1 group was greatly increased with the administration of ferrostatin-1, in comparison with that in ACMP group (Fig. 3F, G). Subsequently, the typical immunostaining images of NF200 and MBP represented that the optical density of NF200 and MBP was significantly increased with the application of ferrostatin-1 than that in ACMP group (Fig. 3H-J). While, the expression of dMBP was evidently downregulated with the treatment of ferrostatin-1 (Fig. 3K, L). Meanwhile, the optical density of dMBP in ACMP + Fer-1 group was markedly decreased in ACMP group (Fig. 3M, N). Taken together, these results demonstrated that mice receiving 10 mg/kg ferrostatin-1 administration facilitated motor recovery through reducing WMI after ACMP.

### 3.4. The application of ferrostatin-1 reduced WMI through repressing ferroptosis after ACMP.

To uncover the reason ferrostatin-1 decreases WMI after ACMP, the administration of ferrostatin-1 on attenuating ferroptosis was explored after ACMP using a series of methods (Fig. 4A). The iron content was evidently decreased with the treatment of ferrostatin-1 from days 3 to 14 (Fig. 4B). Meantime, the Perl’s blue staining images represented that the positive area was remarkably reduced in ACMP + Fer-1 group that was increased in mice after ACMP (Fig. 4C, D). Afterward, the content of MDA in ACMP + Fer-1 group was significantly decreased than that in ACMP group (Fig. 4E). Thereafter, the ROS level was clearly declined with the application of ferrostatin-1 (Fig. 4F, G). Thereafter, the expression of IREB2 was evaluated using western blot assays. The bands indicated that the expression of IREB2 was clearly downregulated with the administration of ferrostatin-1 that was greatly elevated after ACMP (Fig. 4H, I). At the same time, the expression of COX2 was also decreased with the application of ferrostatin-1 after ACMP (Fig. 4J, K). Subsequently, the TEM images showed that the proportion of shrunken mitochondria was clearly abated with the treatment of ferrostatin-1 after ACMP (Fig. 4L, M). Collectively, these results
demonstrated that the administration of ferrostatin-1 reduced WMI by alleviating ferroptosis in mice after ACMP.

3.5. The administration of ferrostatin-1 suppressed ferroptosis via increasing the expression of GPX4 through the nuclear factor erythroid-related factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling pathway after ACMP.

The above results indicated that the administration of ferrostatin-1 mitigated ferroptosis to diminish WMI after ACMP. The underlying mechanism was investigated using western blot and immunofluorescence assays to examine the expression of ferroptosis-associated proteins (Fig. 5A). Firstly, the immunoblot bands demonstrated that the expression of glutathione peroxidase 4 (GPX4), one of the ferroptosis suppressors [40], was obviously downregulated after ACMP, while the administration of ferrostatin-1 partially abrogated this effect that was upregulated the expression of GPX4, to some extent (Fig. 5B, C). In the meantime, the expression of ferritin heavy chain 1 (FTH1), another inhibitor of ferroptosis [41], was evidently decreased, while the application of ferrostatin-1 abolished this effect, in a degree (Fig. 5D, E). However, the expression of ferroptosis suppressor protein 1 (FSP1) showed no significant difference among Sham, ACMP, and ACMP + Fer-1 groups, indicating that FSP1 was not participated in suppressing ferroptosis in mice after ACMP (Fig. 5F, G). Thereafter, the immunostaining of MBP and GPX4 was conducted to certify the results obtained from the western blot assays. The immunostaining images showed that the optical density of GPX4 was significantly increased after ACMP, and this effect was partially abolished with the treatment of ferrostatin-1 (Fig. 5H, J). Meanwhile, the optical density of FTH1 showed the same tendency as GPX4, and the application of ferrostatin-1 clearly increased the optical density of FTH1 (Fig. 5K, L). Together, these results demonstrated that the administration of ferrostatin-1 decreased ferroptosis through upregulating the expression profiles of GPX4 and FTH1.

Previous study has demonstrated that Nrf2/HO-1 signaling pathway is involved in GPX4-regulated ferroptosis in S100-induced experimental autoimmune hepatitis [39]. The expression of Nrf2 and HO-1 was determined using immunoblot and immunofluorescence assays to examine the role of Nrf2/HO-1 signaling pathway in suppressing ferroptosis originating from ferrostatin-1 after ACMP (Fig. 6A). The bands showed that the expression of Nrf2 was slightly upregulated in ACMP group, compared with Sham group (Fig. 6B, C). In the meantime, the expression of Nrf2 was significantly elevated in ACMP + Fer-1 group in comparison with Sham and ACMP groups (Fig. 6B, C). Meanwhile, the immunostaining images of Nrf2 showed that the optical density of Nrf2 was slightly elevated in mice after ACMP, and that was clearly increased with the application of ferrostatin-1 (Fig. 6D, E). Furthermore, the expression of HO-1 was increased in mice after ACMP, and the administration of ferrostatin-1 profoundly increased HO-1 expression, compared with Sham and ACMP groups (Fig. 6F, G). Subsequently, the optical density of HO-1 was profoundly increased in mice after ACMP, and this effect was remarkably enhanced with the administration of ferrostatin-1 using immunohistochemistry assays (Fig. 6H, I). Collectively, these results illustrated that a positive correlation existed between Nrf2 and HO-1, and the application of ferrostatin-1 reduced ferroptosis through Nrf2/HO-1 signaling pathway in mice after ACMP.
4. Discussion

ACMP is one of the most common clinical emergencies and usually leads to intoxication and death worldwide. DEACMP is one of the common delayed complications after ACMP, while the pathogenesis of DEACMP remains unclear. Previous studies provide a clue that WMI might play an evident role in DEAMCP progression following ACMP [10–12]. Whether ACMP causes WMI and the underlying mechanism remains elusive. Here, our study provides evidence that the accumulation of surplus iron and ROS accumulates in WM and initiates WMI due to ferroptosis. And, the administration of ferrostatin-1, one of the ferroptosis inhibitors, reduces iron and ROS deposition to repress ferroptosis, thereafter reducing WMI. Furthermore, the result demonstrated that Nrf2/HO-1 signaling pathway is involved in attenuating ferroptosis with the application of ferrostatin-1 (Fig. 7). The present study offers a rationale that targeting ferroptosis to alleviate WMI is a feasible therapeutic strategy for ACMP and provides a suitable candidate for the treatment of DEACMP.

DEACMP is one of the common delayed complications after ACMP, affecting approximately 30–50% of survivors [4]. The mechanism of secondary brain injury after ACMP is intricated, including CO-originated tissue hypoxia and ischemia, neuronal apoptosis, ROS damage, neurocytotoxicity and neuroinflammatory injury [1, 3]. Hyperbaric oxygen (HBO) therapy has been considered one of the effective treatment options in eliminating COHb to alleviate tissue hypoxia and ischemia after ACMP [42]. However, HBO treatment does not reduce the incidence of DEACMP [43]. The reason for this phenomenon might ascribe to excessive ROS production due to reoxygenation is that patients are removed from CO-enriched environment. Meanwhile, previous studies have indicated that patients with DEACMP show extensive WM lesions arising from the periventricular, occipital, frontal, semiolval centers, and the corpus callosum [10–12]. Oligodendrocytes, the main neural subtype responsible for myelin, are susceptible to ferroptosis [21]. Whether ferroptosis happens at WM to elicit WMI after ACMP is not fully explored. Here, the periventricular brain tissues of the basal ganglia, where are rich in WM [44], were collected and explored the role and mechanism of ferroptosis in WMI after ACMP. To our limited knowledge, it is the first report to certify that ferroptosis evokes WMI after ACMP. Hence, exploring therapeutic regimens suppressing ferroptosis is a feasible approach to reduce WMI after ACMP.

Ferroptosis is characterized by an iron-dependent form of RCD, which occurs through the lethal accumulation of lipid-based ROS [45]. Previous studies have demonstrated that ferroptosis exerts damage to various neurocytes in a series of CNS diseases including stroke (ischemic and hemorrhagic subtypes) [22, 24, 29], traumatic brain injury (TBI) [22, 27, 28], SCI [21] and neurodegenerative diseases [30–32]. Here, our results offered testimony that ferroptosis resulting from iron and ROS accumulation ignited WMI after ACMP, which might bridge the gap between WMI and DEACMP. Meanwhile, the application of ferrostatin-1 reduced iron and ROS deposition, then extenuated WMI through mitigating ferroptosis, which is consistent with previous study in rats after SCI [21]. Furthermore, the administration of ferrostatin-1 protects neurons from ferroptosis-induced damage by attenuating the increased expression level of PTGS2 and its gene product cyclooxygenase-2 (COX2) after intracerebral hemorrhage [46]. In addition, ferrostatin-1 protects astrocytes against angiotensin by suppressing
neuroinflammation and ferroptosis [47]. Additionally, ferrostatin-1 holds the ability of promoting microglial cell polarization from M1 to M2 phenotype to restrain inflammation and to promote hematoma absorption, thereafter improving neurological function in ICH mice [25]. Hence, ferrostatin-1 might exert multiple neuroprotective effects after CNS injury. While, the present study focuses on the administration of ferrostatin-1 in reducing WM after ACMP. The beneficial effects for other neural subtypes need to be elucidated in future research. However, the present work enlarges the therapeutic scope of ferrostatin-1 in treating ACMP.

Nrf2/HO-1 signaling pathway has been proven to be a crucial mediator protecting brain injury against oxidative damage in neurodegenerative diseases [48–50]. Under oxidative stress, Nrf2 could release from Nrf2/Keap1 complex and translocate into the nucleus [48]. With the translocation of Nrf2 into the nucleus, the expression of its downstream effector HO-1 is increased to repress ferroptosis [50, 51]. Here, the results demonstrated that the expression of Nrf2 and HO-1 was slightly increased in a positive correlation after ACMP. The reason for this phenomenon might ascribe to the negative feedback regulation resulting from the increased oxidative stress after ACMP, which is supported by previous report [52]. Meanwhile, the application of ferrostatin-1 evidently upregulated the expression of Nrf2 and HO-1 after ACMP, which is in line with previous studies [48, 53]. FTH1, another downstream mediator of Nrf2, holds the potential of anti-ferroptosis [41]. Here, the results showcased that the expression of FTH1 was evidently decreased, and the administration of ferrostatin-1 clearly reversed this inhibitory effect, further indicating that Nrf2 signaling pathway is involved in ferrostatin-1 suppressing ferroptosis. With the activation of Nrf2 pathway, ferroptosis-related proteins are downregulated such as GPX4 [54], that is in agreement with the findings in the present work. Hence, the Nrf2/HO-1 signaling pathway is a potential therapeutic target for ferroptosis after ACMP. And, ferrostatin-1 could serve as an adjunctive candidate to treat ACMP.

Several limitations need to be clarified in our future research. First, to decipher further signaling pathways underlying the neuroprotective effect of ferrostatin-1, some protein expression with respect to ferroptosis such as glutathione (GSH), glutathione peroxidase 4 (GPX4), nuclear factor erythroid-related factor 2 (Nrf2) and ferroptosis suppressor protein 1 (FSP1), need to be determined. Next, a previous report indicates that p53 upregulation induces ferroptosis in cerebral ischemic penumbra [55]. Meanwhile, TLR4/NF-kB axis is associated with inflammation and oxidative stress to initiate ferroptosis [56]. Given that ferrostatin-1 is an anti-ferroptosis agent, whether ferrostatin-1 could downregulate the expression of p53 and/or reduce the activity of TLR4/NF-kB axis should be elucidated. In addition, several cell death pathways are activated when ROS accumulation, including apoptosis, autophagy, ferroptosis and necrosis. Whether a crosstalk exists among these cell death pathways and which molecule is the trigger are worthy investigating.

5. Conclusion

In conclusion, the present study reveals the novel findings that ferroptosis plays an evident role in WMI after ACMP, and targeting ferroptosis using ferrostatin-1 reduces iron and ROS deposition, downregulates
the expression of IREB2 and COX2 to further attenuate ferroptosis, finally reducing WMI and promoting functional recovery following ACMP in mice. Meanwhile, the results also indicate that Nrf2/HO-1 signaling pathway takes part in suppressing ferroptosis derived from the administration of ferrostatin-1. Collectively, the present study provides evidence that targeting ferroptosis to alleviate WMI is a feasible therapeutic strategy for ACMP and enlarges the therapeutic scope of ferrostatin-1 in the treatment of CNS injury in the presence of ferroptosis.

**Abbreviations**

ACMP acute carbon monoxide poisoning

MRI magnetic resonance imaging

WMI white matter injury

RCD regulated cell death

ROS reactive oxygen species

CNS central nervous system

SCI spinal cord injury

CIS cerebral ischemic stroke

ICH intracerebral hemorrhage

TBI traumatic brain injury

SAH subarachnoid hemorrhage

IREB2 iron responsive element binding protein 2

PTGS2 prostaglandin-endoperoxide synthase 2

LFB Luxol fast blue

OFT open field test

BMT beam walking test

Hb hemoglobin

PBS phosphate-buffered solution

DHE Dihydroethidium
MDA Malondialdehyde

IF immunofluorescent

IHC immunohistochemistry

BSA bovine serum albumin

BCA bicinechinonic acid

TEM transmission electron microscopy

**Declarations**

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**Compliance with Ethical Standards**

**Disclosure of potential conflicts of interest**

The authors have no relevant financial or non-financial interests to disclose.

**Research involving Animals**

All procedures were approved by the local authorities of Zunyi Medical University for the laboratory use of animals (approve no. KLLY(A)-2021-033) and supervised by the Ethics Committee of Affiliated Hospital of Zunyi Medical University. All experimental procedures were performed according to the China's animal welfare legislation for the protection of animals used for scientific purposes.

**Informed consent**

Not applicable.

**Consent to participate**

Not applicable.

**Consent for Publication**

Not applicable.

**Author contributions**
Anyong Yu, Hongfei Ge and Tianxi Zhang designed this research. Shuhong Wang, Binyuan Xiong, Yin Tian, Quan Hu, Xuheng Jiang and Ji Zhang performed the experiments. Mo Li and Xin Zhou analyzed the data. Ruilie Wang and Yuanlan Lu provided technical support. Shuhong Wang wrote a preliminary draft of the manuscript. Hongfei Ge, Anyong Yu, and Tianxi Zhang designed the experiments and revised the manuscript. Anyong Yu made the hypothesis. All authors discussed and approved the final version of the manuscript.

Conflict of interest

The authors declare they have no conflict of interest.

Acknowledge

Not applicable.

Data Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References


Figures
Figure 1

ACMP induced motor deficits due to WMI after ACMP.

(A) Schematic illustration of mouse ACMP model and experimental design. (B) The bar graph indicating the blood CO content in the Sham and ACMP groups. n=5 per group, ***p<0.001; Student’s t test. (C) Mean velocity of mice in each group in open field test. n=8 each group, **p<0.01, ***p<0.001; multiple t tests. (D)
Beam walking test of mice in each group. n=8 each group, ***p<0.001; multiple t tests. (E) Immunoblot bands depicting the expression of MBP in each group. (F) Semi-quantification of the MBP expression from (F). n=4 per group, ***p<0.001; Student’s t test. (G) Luxol fast blue (LFB) staining in Sham and ACMP groups. Scale bars: 20 μm. (H) Bar graph summarizing the optical density of LFB staining from (H). n=7 each group, ***p<0.001; Student’s t test. (I) The representative immunostaining images delineating the expression of NF200 (red) and MBP (green) in each group. Nuclei were counterstained with DAPI. Scale bars: 50 μm. (J, K) Semi-quantitative data showing the optic density of NF200 and MBP in each group from (J). n=7 each group, ***p<0.001; Student’s t test. (L) Immunoblot bands demonstrating the expression of dMBP in each group. (M) Semi-quantification of the dMBP expression from (M). n=4 each group, ***p<0.001; Student’s t test. (N) Immunostaining images demonstrating the expression of dMBP in each group. Nuclei were counterstained with DAPI. Scale bars: 50 μm. (O) Semi-quantitative bar graph indicating the optic density of dMBP in each group from (O). n=7 each group. ***p<0.001; Student’s t test.
Figure 2

ACMP evoked WMI due to ferroptosis.

(A) Schematic illustration of mouse ACMP model and experimental design. (B) Iron concentration in different groups on days 1, 3, 7, and 14. n=3 each group, *p<0.05, **p<0.01, ***p<0.001; multiple t tests. (C) Perl's blue staining images showing the iron accumulation in different groups. Scale bars: 20 μm. (D)
Semi-quantitative data illustrating the percentage of positive area of Perl's blue staining in each group. n=7 per group, ***p<0.001; Student's t test. (E) MDA tests indicating the level of lipid peroxidation. n=7 per group, ***p<0.001; Student's t test. (F) The typical DHE staining demonstrating the ROS levels in each group. Nuclei were counterstained with DAPI. Scale bars: 5 μm. (G) Semi-quantitative bar graph indicating the optic density of ROS from (F). n=7 per group, ***p<0.001; Student's t test. (H) Immunoblot bands depicting the expression of IREB2 in each group. (I) Semi-quantification of the IREB2 expression. n=4 per group, ***p<0.001; Student's t test. (J) Immunoblot bands depicting the expression of COX2 in each group. (K) Semi-quantification of the COX2 expression. n=4 per group, ***p<0.001; Student's t test. (L) The typical TEM imaging showing the morphology of mitochondria in each group. Scale bars: 0.5 μm. (M) The percentage of shrunken mitochondria in each group from (L). n=7 per group, ***p<0.001; Student's t test.
Figure 3

The administration of ferostatin-1 improved motor deficits by reducing WMI after ACMP.

(A) Schematic illustration of mouse ACMP model and experimental design. (B) Survival rate of mice in each group on days 1, 3, 7 and 14. n=8 each group, *p<0.05; Log rank test. (C) Mean velocity of mice in each group in open field test. n=8 per group, **p<0.01, ***p<0.001; multiple t tests. (D) Beam walking test of...
mice in each group. n=8 each group, *p<0.05, **p<0.01, ***p<0.001; multiple t tests. (E) Immunoblot bands depicting the expression of MBP in each group. (F) Semi-quantitation of the MBP expression from (E). n=4 each group, ***p<0.001; Student’s t test. (G) Luxol fast blue (LFB) staining presenting the intensity of WM. Scale bars: 20 μm. (H) Bar graph demonstrating the optical density of LFB staining from (G). n=7 each group, ***p<0.001; Student’s t test. (I) Immunostaining images delineating the expression of NF200 (red) and MBP (green) in each group. Nuclei were counterstained with DAPI. Scale bars: 50 μm. (J, K) Semi-quantitative data showing the optic density of NF200 and MBP in each group. n=7 each group, **p<0.01, ***p<0.001; Student’s t test. (L) Immunoblot bands demonstrating the expression of dMBP in different groups. (M) Semi-quantitative bar graph indicating the optic density of dMBP in each group from (L). n=4 each group, ***p<0.001; Student’s t test. (N) Immunostaining images demonstrating the expression of dMBP in each group. Nuclei were counterstained with DAPI. Scale bars: 50 μm. (O) Semi-quantitative bar graph showcasing the optic density of dMBP in each group. n=7 each group, ***p<0.001; Student’s t test.
**Figure 4**

The application of ferrostatin-1 reduced WMI through repressing ferroptosis after ACMP.

(A) Schematic illustration of mouse ACMP model and experimental design. (B) Iron concentration in different groups on days 1, 3, 7, and 14. n=3 each group, **p<0.01; multiple t tests. (C) Perl’s blue staining images showing the iron accumulation in different groups. Scale bars: 20 μm. (D) Semi-quantitative data
showing the percentage of positive area of Perl’s blue staining in each group from (C). n=7 per group, *** p<0.001; Student’s t test. (E) MDA tests indicating the level of lipid peroxidation. n=7 per group, *** p<0.001; Student’s t test. (F) The typical DHE staining demonstrating the ROS level in each group. Nuclei were counterstained with DAPI. Scale bars: 5 μm. (G) Semi-quantitative bar graph indicating the optic density of ROS in each group from (F). n=7 per group, *** p<0.001; Student’s t test. (H) Immunoblot bands depicting the expression of IREB2 in each group. (I) Semi-quantification of the IREB2 expression from (H). n=4 per group, *** p<0.001; Student’s t test. (J) Immunoblot bands demonstrating the expression of COX2 in each group. (K) Semi-quantification of the COX2 expression from (J). n=4 per group, ** p<0.01; Student’s t test. (L) The typical TEM imaging showing the morphology of mitochondria in each group. Scale bars: 0.5 μm. (M) The percentage of shrunken mitochondria in each group from (L). n=7 per group, *** p<0.001; Student’s t test.
Figure 5

The administration of ferrostatin-1 attenuated ferroptosis via increasing the expression of GPX4 and FTH1 after ACMP.

(A) Schematic illustration of mouse ACMP model and experimental design. (B) Immunoblot bands depicting the expression of GPX4 in each group. (C) Semi-quantification of the GPX4 expression from (B).
n=4 each group, *p<0.05, ***p<0.001; one-way ANOVA, followed by Tukey's post hoc test. **(D)** Immunoblot bands indicating the expression of FTH1 in each group. **(E)** Semi-quantification of the FTH1 expression from (D). n=4 each group, *p<0.05, **p<0.01, ***p<0.001; one-way ANOVA, followed by Tukey's post hoc test. **(F)** Immunoblot bands showing the expression of FSP1 in each group. **(G)** Semi-quantification of the FSP1 expression from (F). n=4 each group, NS: no statistical difference; one-way ANOVA, followed by Tukey's post hoc test. **(H)** The representative immunostaining images disclosing the expression of GPX4 (red) and MBP (green) in each group. Nuclei were counterstained with DAPI. Scale bars: 50 μm. **(I, J)** Semi-quantitative data showing the optic density of GPX4 and MBP in each group from (H). n=7 each group, *p<0.05, **p<0.01, ***p<0.001; one-way ANOVA, followed by Tukey's post hoc test. **(K)** Immunohistochemistry images indicating the expression of FTH1 in different groups. Scale bars: 20 μm. **(L)** Semi-quantitative data showing the optic density of FTH1 in each group from (K). n=7 each group. ***p<0.01, ***p<0.001; one-way ANOVA, followed by Tukey's post hoc test.**
Figure 6

The application of ferrostatin-1 inhibited ferroptosis through mediating Nrf2/HO-1 signaling pathway after ACMP.

(A) Schematic illustration of mouse ACMP model and experimental design. (B) Immunoblot bands presenting the expression of Nrf2 in each group. (C) Semi-quantification of the NRF2 expression from (B).
n=4 each group, *p<0.05, **p<0.01; one-way ANOVA, followed by Tukey's post hoc test. (D) Representative immunostaining images demonstrating the expression of NRF2 in different groups. Scale bars: 50 μm. (E) Semi-quantification of the Nrf2 expression from (D). n=7 each group, **p<0.01, ***p<0.001; one-way ANOVA, followed by Tukey's post hoc test. (F) Immunoblot bands depicting the expression of HO-1 in each group. (G) Semi-quantification of the HO-1 expression from (F). n=4 each group, *p<0.05, ***p<0.001; one-way ANOVA, followed by Tukey's post hoc test. (H) Immunohistochemistry images representing the expression of HO-1 in different groups. Scale bars: 20 μm. (I) Semi-quantification of the HO-1 expression from (H). n=7 each group, *p<0.05, **p<0.01, ***p<0.001; one-way ANOVA, followed by Tukey's post hoc test.

Figure 7

Schematic illustration demonstrating that targeting ferroptosis mitigated WMI to facilitate functional recovery and the underlying mechanism following ACMP.