Deferoxamine Reduces Endothelial Ferroptosis and Protects Cerebrovascular Function after Experimental Traumatic Brain Injury

Yidan Liang  
Department of Neurosurgery, Chongqing University Central Hospital, Chongqing Emergency Medical Center

Yanglingxi Wang  
Department of Neurosurgery, Chongqing University Central Hospital, Chongqing Emergency Medical Center

Chao Sun  
Department of Neurosurgery, Chongqing University Central Hospital, Chongqing Emergency Medical Center

Yi Xiang  
Department of Neurosurgery, Chongqing University Central Hospital, Chongqing Emergency Medical Center

Yongbing Deng  
Department of Neurosurgery, Chongqing University Central Hospital, Chongqing Emergency Medical Center

Research Article

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Abstract

Cerebrovascular dysfunction resulting from traumatic brain injury (TBI) significantly contributes to poor patient outcomes. Recent studies revealed the involvement of iron metabolism in neuronal survival, yet its effect on vasculature remains unclear. This study aims to explore the impact of endothelial ferroptosis on cerebrovascular function in TBI. A Controlled Cortical Impact (CCI) model was established in mice, resulting in a significant increase in iron-related proteins such as TfR1, FPN1, and FTH, as well as oxidative stress biomarker 4HNE. This was accompanied by a decline in expression of the ferroptosis inhibitor NCOA4. Moreover, Perls' staining and nonhemin iron content assay showed iron overload in brain microvascular endothelial cells (BMECs) and the ipsilateral cortex. Immunofluorescence staining revealed more FTH-positive cerebral endothelial cells, consistent with impaired perfused vessel density and cerebral blood flow. As a specific iron chelator, DFO treatment inhibited such ferroptotic proteins expression and the accumulation of lipid-reactive oxygen species following CCI, enhancing glutathione peroxidase (GPx) activity. DFO treatment significantly reduced iron deposition in endothelial cells and brain tissue, and increased density of the cerebral capillaries as well. Consequently, DFO treatment led to improvements in cerebral blood flow (as measured by laser speckle imaging) and behavioral performance (as measured by the NSS scoring, rotarod test, and Morris water maze test). Taken together, our results indicated that TBI induces remarkable iron disorder and endothelial ferroptosis, and DFO treatment may help maintain iron homeostasis and protect vascular function. This may provide a novel therapeutic strategy to prevent cerebrovascular dysfunction following TBI.

Introduction

Traumatic brain injury (TBI) is a significant public health issue associated with high morbidity and mortality globally, resulting in long-term changes incognitive and physiological functions of the survivors [1]. The cerebrovascular exerts crucial effects on providing blood to the brain. As reported in experimental animals and patients, TBI leads to cerebrovascular dysfunction, characterized by endothelial impairment and reduced cerebral perfusion, leading to a cascade of secondary injuries such as blood-brain barrier (BBB) breakdown, edema, hemorrhage, and coagulopathies, which exacerbate lasting neurological deficiencies [2–3]. Unfortunately, there are currently few effective treatments available to mitigate these conditions, emphasizing the need for new treatment approaches.

As the main component of vessel lining, endothelial cells serve as a barrier between blood and surrounding tissues, are essential in regulating cerebrovascular function by controlling blood vessel tone, blood flow, and coagulation [4]. Previous research has demonstrated that endothelial cell dysfunction leads to cell death and subsequent impairment of cerebral perfusion in TBI [5–6]. However, the precise physiopathologic mechanisms have yet to be fully understood.

Iron homeostasis is vital for maintaining cellular integrity and regulating various biological processes, including oxygen transport, neurotransmission, myelination, and neuronal metabolism. Ferroptosis, a newly reported programmed cell death, happens when high levels of toxic reactive oxygen species (ROS)
lead to fatal lipid peroxidation due to abnormal deposition of iron [7]. Some researchers have highlighted
the involvement of ferroptosis in the pathophysiologic mechanism of several neurological diseases such
as Alzheimer’s disease, subarachnoid hemorrhage (SAH), ischemic stroke and Parkinson’s disease [8–10].
While ferroptotic death and the remarkable effects of iron metabolism on neuronal survival after TBI has
been revealed recently [11–12], the role of ferroptosis in the vasculature has yet to be fully elucidated.

As for TBI treatment, solely neuroprotective strategies was confirmed to be clinically limited [13]. Hence,
our study aimed at providing new evidence for enhancing microvascular perfusion. In this context, we
explore the iron metabolism of brain endothelial cells and its effect on cerebral perfusion by performing a
mice CCI model. Mechanistically, our results reveal that deferoxamine (DFO), a potent iron chelator agent,
prevents brain endothelial cells from TBI-induced iron dysregulation, reducing accumulation of ROS and
subsequent oxidative injury, thus rescuing endothelial cell ferroptosis and perfusion impairment. DFO, the
potential anti-ferroptotic drug, may be a promising therapeutic target for TBI treatment.

Materials and methods

Animals

Male C57BL/6 mice weighing between 25 and 30 grams and aged 12 weeks were procured from the
Experimental Animal Center of Chongqing Medical University, and all animal experiments were conducted
in accordance with the National Institutes of Health guidelines for the care and use of animals. We kept
the mice under standardized light/dark cycle (12/12-hour), humidity (60 ± 5%), and temperature (22 ±
3°C), and they were free to food and water. Mice distribution were randomly performed, and all
procedures were approved by the Ethics Committee of the Chongqing Emergency Medical Center. The
mice were anesthetized using 3% isoflurane and kept under anesthesia with a mixture of 1.5% isoflurane
(Zhong et al., 2017).

Mouse TBI Model and Drug Administration

To induce a moderate to severe contusion, we established a CCI model, following established protocols
[14–15]. As previously described, the parameters were set as follows: 1) velocity: 5.0 m/second; 2) depth:
2.0 mm; 3) dwelling time: 100 ms [16]. As for the sham group, mice underwent craniotomy without CCI.
Brain tissue samples were collected from a subset of mice to isolate brain microvascular endothelial cells
(BMECs) at different time points after CCI (6 hours, 1st, 3rd, 7th, and 14th days).

Drug Administration

Deferoxamine (DFO), a highly specific iron chelator, was procured from Sigma-Aldrich. After the surgery,
100 mg/kg DFO was administered intraperitoneally every 12 hours, lasting for 7 days, along with the
vehicle control (0.5% DMSO in saline, ip) [17–18].

Isolation of Brain Microvascular Endothelial Cells (BMECs)
In accordance with previously published BMECs isolation protocols [19–20], the brains were perfused with pH 7.4 PBS and swiftly separated into two hemispheres, the ipsilateral cortex was homogenized in ice-cold PBS using a Knote Dounce glass tissue grinder. After centrifugation for 5 minutes (1000×g, 4°C), discarded the supernatant, then resuspended the deposit with an 18% Dextran solution in PBS. Following another centrifugation for 15 minutes (2000×g, 4°C), washed the vessel deposit after discarding the supernatant again, and then. Finally, the remaining brain microvessel fragments, which are purified BMECs, were leached by a 40 µm cell filter, in order to remove the debris and were used for further processing and measurements.

**Western Blotting Assay**

The western blot analysis was conducted following established protocols [10, 21]. Total protein was extracted from collected BMECs. Briefly, 0.2g of cortical tissue from each group was taken in separate EP tubes, a mixture of tissue lysis solution and protease inhibitor was added. The tissue was then smashed and standed for 20 minutes. Centrifugating for 15 minutes (12,000×g, 4°C) to get the supernatant, then measured its concentration. Different samples were then added to the gel, after gel electrophoresis, they were separated and transferred to PVDF membranes (Millipore, Boston, MA, USA). Then blocked the membranes with 5% non-fat milk for 1 hour and incubated them overnight with primary antibodies (4°C). The following primary antibodies were used: anti-GAPDH (1:1000,Cat# 60004-1-Ig, Proteintech, Wuhan, China), anti-transferrin receptor 1 (TfR1; 1:1000, Cat# SAB4200398, Sigma-Aldrich, USA), anti-ferroportin1 (FPN1; 1:1000, Cat# MTP11-S, Alpha Diagnostic International, USA), anti-ferritin heavy chain (FTH; 1:10,00, Cat# ab183781, Abcam, USA), anti-4-hydroxynonenal (4-HNE; 1:1000, Cat# ab46545, Abcam, USA), and anti-GPX4 (1:1000, Cat# 14432-1-AP, Proteintech, China). After being incubated with secondary antibody (1:5000; Cat# SA00001-2, Proteintech, China) and ECL reagent (ECL Plus, Millipore), the membranes were quantified with the Fusion system (Fusion, France).

**Immunofluorescence Staining**

Immunofluorescence staining was performed on day 3 following TBI, and was carried out according to established procedures [22]. The mouse brains were extracted after transcardial perfusion with PBS and 4% paraformaldehyde, and then fixed with 4% PFA for 24 hours (4°C). The brains were cut into 10 µm-thick slices, which were subsequently postfixed, dehydrated, and keeped at -80°C. Incubating the slices with primary antibodies overnight after blocking with PBS, the primary antibodies are as follows: anti-CD31 (1:100, Cat# AF3628, Novus, USA) and anti-ferritin heavy chain (FTH; 1:100, Cat# ab183781, Abcam, USA). Next, the slices were incubated with secondary antibodies conjugated with red and green fluorescein (1:250, Thermo Fisher Scientific, USA) for 1 hour. Finally, the slices were stained with DAPI. The fluorescence signals were visualized by an Olympus inverted microscope (BX51; Olympus, USA).

**Perls’ Prussian Blue Staining**

The protocol for Perls' prussian blue staining was carried out following the previous studies [23–24]. Seven days after TBI, sacrificed the mice, quickly removed the brains and cut into 10 µm-thick paraffin slices. Next, sequentially put them in xylene I and xylene II for 15 minutes each. The tissue underwent
gradient hydration using solutions of increasing ethanol concentration (100%, 95%, 85%, 80%, 75%, and 70%). Next, incubated the slices with the Perls' staining solution for 1 minute, and after washing with PBS, the slices were subjected to HE staining for 1 minute, and underwent rapid gradient dehydration using solutions of increasing ethanol concentration (80%, 85%, 90%, and 100%) for 5 seconds each time. Finally, fixed and sealed the slices.

**Non-heme Iron Content Determination**

Ferroptosis has been associated with alterations in intracellular and tissular iron accumulation, depleted glutathione (GSH) levels, malondialdehyde (MDA) content, and glutathione peroxidase (GPx) activity [7, 25]. The content of Fe$^{2+}$ and Fe$^{3+}$ in the ipsilateral microvessels were detected by an assay kit (Cat# ab83366, Abcam, USA) refer to the manufacturer's suggestions three days after TBI. Brain microvessel fragments were first weighed, homogenized in iron assay solution, after centrifuging for 10 minutes (16, 000×g, 4 °C), the supernatant was collected. Next, 5 µL of assay buffer and iron reducer were added. Finally, incubated the mixture for 30 minutes(37°C), and the results were obtained at 593 nm using a colorimetric microplate reader (Thermo, USA). The Fe$^{3+}$ level of the samples were calculated as the formula: Fe$^{3+}$ = Total iron - Fe$^{2+}$.

**GSH/GSSG Assay**

One day after TBI, GSH content in the ipsilateral microvessels was measured by an assay kit (Cat# S0053, Beyotime Biotechnology, China) following the instructions. The results were obtained at 593 nm using a colorimetric microplate reader mentioned previously. GSH content was determined using the formula: Total Glutathione-GSSG × 2.

**MDA Assay**

Malondialdehyde (MDA) levels in ipsilateral microvessels were assessed 3 days post-TBI by an assay kit (Cat# S0131S, Beyotime Biotechnology, China). Brain microvessel fragments from ipsilateral hemisphere were first weighed and homogenized. Then the supernatant was collected after centrifuging for 10 minutes (10,000–12,000×g, 4 °C). Next, the MDA solution was added to the samples and standard well. Finally, heated the mixture and collected the supernatant after centrifuging the mixture for another 10 minutes (1000×g, 4 °C). MDA levels were obtained at 532 nm using a colorimetric microplate reader mentioned previously.

**Glutathione Peroxidase (GPx) Activity Assay**

The GPX4 enzyme is responsible for eliminating harmful forms of ROS in the brain. Inhibition of GPX4 is one of the key triggers of ferroptosis. Thus, GPX4 is regarded as a marker of ferroptosis. To detect the GPx activity in ipsilateral microvessels, a GPx assay kit (Cat# S0056, Beyotime Biotechnology, China) was used three days after TBI. The results were obtained at 340 nm by the previously mentioned colorimetric microplate reader.

**Quantification of Blood Vessel Density**
After 28 days following TBI, 300 µL of lectin dye (Cat# FL-1171, Vector Laboratories) was administered via the tail vein, after circulating for 10 minutes, the mice were sacrificed. The fluorescence signals in injury brain section were visualized under a fluorescence microscope as previously mentioned.

### Cerebral Blood Flow Analysis

Laser speckle contrast analysis (LASCA) was performed on day 7 post-TBI according to the previous studies [26–28]. Briefly, the indicator laser was adjusted to the center part of the injury brain section. The signals of cerebral blood flow were obtained at 785 nm and a CCD camera was used to obtain the perfusion images.

### Measurements of Neurological Functions

The neurobehavior of mice was evaluated according to previous studies [29] by the neurological severity scores (NSS). The NSS is a indicator of neurological outcome for the mice at various time points, on the timepoint of before injury, as well as on the 1st, 3rd, 7th, 14th, 21st, and 28th days post-TBI. The evaluation comprised several aspects of mouse behavior, such as general behavior, balance, alertness, and motor ability, by conducting ten different tasks. Points were assigned for each failed task, with a higher score indicating a more severe neurological deficit and a worse outcome. Additionally, the mice's motor abilities were examined by using the rotarod test, which supposed to measure their ability for keeping balance and then remaining on a rotating rod. Besides, we performed the Morris water maze test to assess the cued learning ability and memory of the mice, and their memory was tested by removing the hiding platform 21 days after TBI.

### Statistical Analysis

The data were analyzed by SPSS 19.0 and the results were presented as mean ± SEM. We used One-way ANOVA and two-way ANOVA to analyze the results, followed by Tukey's post hoc test to determine where the differences lay. The p-value less than 0.05 was considered to be statistical significance. The data was plotted by GraphPad Prism 9.0 software.

### Results

#### Iron Dysregulation and Oxidative Stress in BMVECs after TBI

As is known, iron metabolism is crucial for maintaining the stability of cellular structure and function, and dysregulation of iron has been shown to contribute to poor outcomes in cases of traumatic brain injury (TBI) [12, 30]. To better understand the effects of TBI on iron metabolism, we analyzed the expression of various ferroptosis-related proteins over time, including TfR1, FPN1, FTH, 4HNE and GPX4. We observed that the expression of TfR1, a protein involved in iron uptake, significantly increased and peaked 24 hours after TBI (Fig. 1A, 1B), suggesting that TBI stimulates iron transportation from the blood into the cytosol of endothelial cells. Additionally, levels of FPN1, the only cellular iron exporter, increased in brain
microvascular endothelial cells (BMECs) after TBI (Fig. 1A, 1C), possibly as a feedback mechanism to combat iron overload. FTH, a cytosolic iron indicator, and 4HNE, a lipid oxidation product and oxidative stress biomarker, were also upregulated 3 days after TBI, with a decline from 3 days to 14 days (Fig. 1A, 1D-E). Interestingly, GPX4, an important protein that regulates ferroptosis, gradually decreased from 1 day to 3 days post-TBI (Fig. 1A, 1F). Taking these results together, our findings reveal that TBI disrupts iron homeostasis and leads to ferroptosis in BMECs.

**DFO Treatment Rescues TBI-Induced Iron Dysregulation and Oxidative Stress in BMECs**

Deferoxamine (DFO), a selective iron chelator, has been proven to safeguard cells away from ferroptosis [17, 31]. To investigate the potential of DFO in mitigating ferroptosis induced by TBI, we performed Western blot analysis to assess levels of the ferroptosis-related proteins mentioned previously at the time points corresponding to their expression peaks after TBI (1 day and 3 days). We observed that DFO treatment suppressed the upregulation of TfR1 and FPN1 proteins induced by TBI at 1 day post-injury (Fig. 2A-C). Furthermore, DFO treatment attenuated the protein levels of FTH and 4HNE, while increasing the expression of GPX4 at 3 days after TBI (Fig. 2D-G). These findings reveal that DFO's iron chelation property can prevent TBI-induced iron accumulation, thereby blocking the production of ROS and oxidative damage. We also evaluated several other potential biomarkers of ferroptosis, including glutathione (GSH) content, malondialdehyde (MDA) levels, and GPX4 activity. GSH is a critical regulator of ferroptosis, and decreased GSH levels can trigger ferroptosis. As expected, DFO treatment attenuated the TBI-induced reduction of GSH levels in BMECs (Fig. 2H). The increase in MDA content, a by-product of lipid peroxidation that characterizes ferroptosis, was significantly suppressed by DFO administration (Fig. 2I). Furthermore, GPX4 activity was reduced after TBI, but DFO treatment remarkably elevated this parameter in BMECs (Fig. 2J). In summary, our results indicate that DFO can prevent TBI-induced iron dysregulation in BMECs, thereby inhibiting ROS production and oxidative injury.

**DFO Treatment Attenuates Iron Accumulation in BMECs and Ipsilateral Tissue after TBI**

To corroborate the effect of DFO on iron aggregation subsequent to traumatic brain injury (TBI) ulteriorly, we performed non-heme iron content analysis, Perls’ prussian blue staining, and immunofluorescence staining experiments. The level of Fe$^{2+}$ denotes iron excess and consequent production of reactive hydroxyl radicals via the Fenton reaction. Our findings indicate a substantial rise in the total iron content, Fe$^{3+}$, and Fe$^{2+}$ after TBI, whereas DFO administration significantly decreased iron accumulation in ipsilateral BMECs three days post-TBI (Fig. 3A). Furthermore, Perls’ blue staining unveiled a marked decrease in free iron deposition in the ipsilateral cortex of DFO-treated mice relative to vehicle-treated mice seven days after TBI (Fig. 3B). Ferritin is the form of cellular iron storage, and TBI-triggered iron overload is accompanied by elevated FTH expression [32]. Hence, using immunofluorescence staining, we assessed the FTH expression in brain endothelial cells three days after TBI. We found an increase in
FTH-positive cerebral endothelial cells in the vehicle group relative to the sham group, however, DFO treatment lessened the ratio of FTH-positive brain endothelial cells in the DFO-treated group in comparison with the vehicle group (Fig. 3C). These findings indicate that DFO inhibits iron accumulation in brain endothelial cells following TBI, which subsequently mitigates oxidative stress.

**DFO Administration Enhances Cerebral Functional Vessel Density and Cortical Perfusion after TBI**

To explore the possibility of DFO on enhancing cerebrovascular function at the delayed phase post-TBI, we performed lectin dye staining on day 28 and cerebral blood flow detection on day 7. Our findings revealed that DFO administration notably increased microvessel density and cerebral perfusion in the ipsilateral cortex after TBI (Fig. 4A-B). These findings suggest that DFO treatment significantly improves cerebral blood flow and increases functional microvessel density in the ipsilateral cortex of mouse brains affected by TBI.

**DFO Infusion Improves Neurological Outcomes of TBI Mice**

To explore whether DFO treatment could improve the neurobehavioral outcomes induced by TBI, we conducted behavioral analyses including the NSS scoring, rotarod test, and the MWM test. The outcome of NSS scoring was significantly declined in TBI group, while treatment with DFO improved neurological performance from day 7 to day 28 after TBI (Fig. 5A). With respect to the rotarod test, DFO administration remarkably improved sensorimotor function from the third day to 28th days post-TBI in comparison with the vehicle group (Fig. 5B). The MWM test was performed on 16 days post-TBI. Mice in the TBI group exhibited more profound learning disorders, as indicated by their prolonged search time and longer travel distance to reach the platform during training. However, DFO administration apparently diminished the latency and distance for finding the platform (Fig. 5C-E).

**Discussion**

Traumatic brain injury (TBI) is a foremost cause of mortality and disability worldwide. The burden of TBI is not only significant for the affected individuals, but also for their families, communities, and society at large due to the long-term care and support required, as well as the substantial economic costs. TBI pathophysiology is characterized by the interplay between primary cellular and tissular damage and the consequent responses induced by the trauma, which can occur over extended time frames. These secondary responses, including BBB breakdown, edema, hemorrhage, and coagulopathies, can all result in poor neurological outcome [13, 33]. The failure of solely neuroprotective strategies in the treatment of TBI has prompted a shift in research focus from neurocentric dysfunctions to the neurovascular unit (NVU) concept. This concept emphasizes the interconnectedness of brain cells and blood vessels for optimal brain function, and highlights the importance of cerebrovascular function on neurological outcomes improvement after TBI [34].
Clinical observations have shown that TBI patients experience decreased cerebral blood flow (CBF) and impaired cerebrovascular autoregulation, along with vasospasms and edema formation caused by subarachnoid hemorrhages [35–37]. Studies indicate that CBF values in TBI patients can be as low as 22.5mL/100g/min within 6 hours after injury, which is significantly below the normal range of 45-50mL/100g/min. CBF reduction improves as recovery occurs, and normalization of CBF within three weeks is associated with better neurological outcomes [38]. These findings emphasize the importance of developing new treatment paradigms aimed at improving cerebrovascular function and recovering cerebral perfusion in the early phase following TBI.

In recent decades, there has been a greater understanding of the changes that can occur within the cerebrovascular and neurovascular unit (NVU) systems following traumatic brain injury (TBI) in preclinical models. The NVU refers to the complex interactions between neurons, astrocytes, endothelial cells, and pericytes, and is a functional unit critical for maintaining neurovascular coupling, which ensures the brain receives an adequate supply of oxygen and nutrients while removing waste products [39]. Endothelial cells, as a key component of the NVU, play a critical role in regulating cerebrovascular function by controlling blood flow, blood coagulation, BBB integrity, and the brain's immune response. They form the barrier between the blood and surrounding tissue, and are able to sense and respond to changes in blood flow and pressure. Additionally, they can release vasoactive substances that can dilate or constrict blood vessels, thereby affecting blood flow and pressure in the brain. As such, our focus has been on the metabolism and function of endothelial cells in addressing cerebrovascular dysfunction.

Ferroptosis is a form of cell death that occurs due to the iron overload and accumulation of ROS and the resulting lipid peroxidation (LPO). The metabolism of brain is quite rapid and it is particularly susceptible to injury from oxidative stress, which is mediated by ROS. Previous studies have clarified the involvement of ferroptosis in the pathophysiologic mechanism of several neurological disorders including traumatic brain injury [24, 30, 40]. Some researchers have suggested that neuronal ferroptosis may be an important mechanism of neurological deficit in TBI [17, 31]. Considering that endothelial cells are especially vulnerable to injury due to their location and exposure to blood flow, we hypothesized that after a traumatic brain injury, being exposed to a complex array of insults, the brain's vasculature became compromised, leading to reduced blood flow phenomenally and increased abnormal iron accumulation, to the molecular aspect. This iron dysregulation resulted in the generation of excessive ROS, which lead to lipid peroxidation, leading to ferroptotic death of the endothelial cells. The loss of endothelial cells subsequently decreased vasodilation and increased vasoconstriction, contributing to cerebrovascular dysfunction and decreased blood flow in the brain.

To further verify our assumption, we performed the CCI model and explored the time-course changes of ferroptosis-related protein expressions in the ipsilateral brain endothelial cells. Moreover, we detected oxidative stress biomarkers such as GSH content, MDA content and GPx activity by assay kits. These results strongly support our hypothesis that TBI affects iron homeostasis and ultimately leads to brain endothelial ferroptosis. Deferoxamine (DFO) is a specific chelator of iron, has been shown to safeguard cells away from ferroptosis resulted by a variety of agents, including GPX4 inhibitors, lipid peroxidation
inducers, and pro-inflammatory cytokines. In addition, DFO has also been shown to protect cells from ferroptotic death of many different models, including some neurological disorder models. However, its effect on brain endothelial ferroptosis and cerebral perfusion remains unclear. Herein, we found that DFO administration rescued iron dysregulation and oxidative stress in brain endothelial cells caused by TBI, reduced iron deposition in BMECs and the ipsilateral cortex, as well as enhancing cerebral functional vessel density and cortical perfusion, ultimately improved neurological outcomes.

There are still some limitations of our research. Firstly, we focused on the role of brain endothelial cells and their iron metabolism on cerebrovascular function, however, the neurovascular unit (NVU) is a complex system and includes other components such as astrocytes and pericytes. Further researches are needed to better understand the mechanism and interactions of these components. Additionally, more investigations are needed to gain a deeper understanding of the cerebrovascular effects of iron metabolism and DFO administration. Finally, while DFO has been shown to reduce injury severity and improve outcomes in various animal models, it is important to conduct careful preclinical evaluations of all the potential translational aspects of DFO.

Declarations

Author Contribution

Yidan Liang, Yanglingxi Wang, Chao Sun and Yi Xiang carried out experiments and collected the data. Yidan Liang and Yongbing Deng performed the statistical and paper writing.

Conflicts of Interest

The authors declare that there are no conflicts of interest associated with this manuscript.

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References


Figures
Figure 1

Traumatic brain injury (TBI) accounts for iron dysregulation and oxidative stress in ipsilateral brain endothelial cells. (A) Time course expression of ferroptosis-related proteins such as TfR1, FPN1, FTH, 4HNE and GPX4 in ipsilateral BMVECs after TBI. (B-F) Relative intensity of proteins in panel. The results are presented as the mean ± SEM (n=6 mice/per group). * p < 0.05, ** p < 0.01, *** p< 0.001, **** p<
0.0001, ns: not statistically significant. TfR1, transferrin R1; FPN1, ferroportin1; FTH, ferritin heavy chain; 4HNE, 4-hydroxynonenal; GPX4, glutathione peroxidase 4;

Figure 2

The effects of DFO treatment on TBI-induced ferroptosis. Western blot for TfR1 and FPN1 was performed at 1 day after TBI, and FTH, 4HNE and GPX4 was performed at 3 days after TBI. (A-G) Representative gel
images of western blotting and quantitative analysis for these proteins. (H) The GSH content was measured in the ipsilateral brain vessel fragments at 1 day following TBI. (I-J), MDA content and GPx activity were measured in the ipsilateral BMECs at 3 days following TBI. Data are expressed as mean ± SEM (n = 6 mice/per group). *P < 0.05, **P < 0.01, and ***P < 0.001, **** p < 0.0001, ns: not statistically significant. MDA, malondialdehyde; GSH, glutathione.
DFO rescued iron accumulation in the ipsilateral cortex endothelial cells after TBI. (A) Endothelial non-heme iron was measured in sham, CCI+Vehicle and CCI+DFO group. *P < 0.05, **P < 0.01, and **** p< 0.0001, ns: not statistically significant. Data are expressed as mean ± SEM (n = 6 mice/per group)(B) Representative images of Perls' blue staining in ipsilateral cortex at 7 days after TB. The scale bar = 50 μm, n = 6 mice/per group). (C) Representative images of co-expression of FTH with markers of endothelial cells (CD31) in brain sections at 3 days following TBI. Original magnification 400× (The scale bar = 50 μm, n = 6 mice/per group).
Figure 4

(A) Functional vessel density was identified by the staining of perfused lectin at 28 days following CCI. Functional vessel density was significantly elevated in the DFO treatment group compared with the CCI group. Original magnification 20×, the scale bar = 50 μm. (B) Effect of DFO on perfusion efficiency at the injury site 7 days following CCI, which was represented by two-dimensional laser speckle images of CBF changes. CBF was significantly enhanced in the CCI+DFO group compared with the CCI group. Circles indicate the CBF in lesion areas.
Figure 5

DFO administration improves neurological outcomes after TBI in mice. (A) Modified neurological severity score (NSS). (B) Rotor rod test. *p < 0.05 compared to CCI + Vehicle at the same time point, data are presented as mean ± SEM (n = 5 mice/per group). (C-D) Latency to platform and distance to platform in the visible platform testing. (E) Representative swimming tracks of the mice in all three groups on the 6th day of the MWM task.*P < 0.05, **P < 0.01, ***P < 0.001 and **** p < 0.0001 compared to Sham, #p < 0.05,
##P < 0.01 and ####p < 0.0001 compared to CCI + Vehicle. Data are presented as mean ± SEM (n = 6 mice/per group)