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Research paper

# Acute iron overload aggravates blood-brain barrier disruption and hemorrhagic transformation after transient focal ischemia in rats with hyperglycemia

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ABSTRACT

Hemorrhagic transformation (HT) has been reported to be associated with a poor prognosis after acute ischemic stroke. Blood-brain barrier (BBB) damage is considered as the major pathophysiologic mechanism of HT. Our aim was to investigate the role of acute iron overload in BBB damage and HT after transient focal ischemia in rats with hyperglycemia. Transient middle cerebral artery occlusion (MCAO) was induced in rats with hyperglycemia. Animals were assigned to four groups: Sham, Vehicle, Iron overload and Iron chelator treatment groups. Brain samples were collected at 24 h after surgery to quantify the amount of hemorrhage, determine extravasation of Evans blue and detect the levels of following proteins: ferritin, matrix metalloproteinase-9 (MMP-9), zonula occludens-1 (ZO-1), Occludin and Claudin-5 by western blot analysis and immunohistochemistry. Compared to the Vehicle group, the Iron overload group had a significantly higher amount of hemorrhage and more extravasation of Evans blue. The Iron overload group had lower levels of ZO-1, Occludin and Claudin-5 and higher levels of ferritin and MMP-9 than the Vehicle group. Administering iron chelator reduced the extension of hemorrhage and extravasation of Evans blue, reversed the MCAO-induced reduction of ZO-1, Occludin and Claudin-5 and decreased the levels of ferritin and MMP-9. Our results suggest that acute iron overload aggravates BBB damage and HT after transient ischemia in rats with hyperglycemia, which provides basic evidence for iron overload as a potential factor associated with BBB damage and HT in ischemic stroke patients when accompanied with hyperglycemia.

## Introduction

Stroke is the second leading cause of morbidity and mortality worldwide adversely affecting patients, their families and societies, of which ischemic stroke accounts for approximately 70%(Krishnamurthi et al., 2020; S. Wu et al., 2019). Over 1/3 of all ischemic stroke patients are accompanied by acute hyperglycemia on admission(Paciaroni et al., 2009). It is widely accepted that hyperglycemia is associated with secondary injuries after acute ischemic stroke such as hemorrhagic transformation (HT)(Couret et al., 2018; W. A. Li et al., 2013; Paciaroni et al., 2009). Several studies have reported that patients with HT have a poor functional outcome and increased mortality after cerebral infarction (van Kranendonk et al., 2019). Therefore, it is necessary to investigate the mechanisms of HT when accompanied with hyperglycemia.

Blood-brain barrier (BBB) breakdown is an important pathophysiologic mechanism of HT after ischemia/reperfusion (I/R) injury(Bernardo-Castro et al., 2020), which can be evaluated by measuring extravasation of Evans blue, and detecting the levels of tight junctions (TJs) including zonula occludens-1 (ZO-1), Occludin and Claudin-5. Although many studies explored underlying molecular targets of BBB

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damage to seek appropriate neuroprotective drugs, no effective drugs for preserving BBB integrity and preventing HT are available in clinical practice yet. In rat I/R models(Bauer et al., 2010), activation of matrix metalloproteinase-9 (MMP-9) was found to be related to the alteration of BBB permeability and degradation of TJs. Our previous studies indicated that MMP-9 may be an important predictor for HT after acute ischemic stroke(Yuan et al., 2018; Zheng et al., 2019).

In cardiac damage(Puukila et al., 2015) and head and neck squamous cell carcinoma(Kaomongkolgit et al., 2008), MMP-9 has been found as one of the downstream molecules regulated by iron overload. Ferritin, a 24-subunit protein complex composed of ferritin heavy chain (FTH) and ferritin light chain (FTL), could be regarded as a reliable index to evaluate status of iron overload(Garcia-Yebenes et al., 2012; Poggiali et al., 2012), which could be attenuated by deferoxamine (DFX), one of the most common iron chelators(Poggiali et al., 2012). Clinical studies have shown that iron overload (measured as increased expression of serum ferritin) can be regarded as an important biomarker associated with HT in the acute phase of ischemic stroke (Choi et al., 2012; Mehrpour and Mehrpour, 2016; Millan et al., 2007), which lacks adequate experimental evidence(Garcia-Yebenes et al., 2018), however.

Thus, we performed a transient focal ischemia model in rats with hyperglycemia to investigate the role of acute iron overload in BBB damage and HT.

## Material and methods

### *Animals and Transient Middle Cerebral Artery Occlusion (MCAO)*

Adult male healthy Sprague–Dawley rats (n = 150) weighing 220–280 g were purchased and had free access to water and food with a 12 h dark/light cycle lasting for 1–2 week before surgery. Anesthesia was induced by inhalation of 5% isoflurane and 2% isoflurane was maintained during surgery. Rectal temperature was maintained at 37.5 ± 0.5 ℃ via a feedback-controlled heating system. MCAO was induced according to Chou's method(Chiang et al., 2011) by a professional staff. All rats received an injection of 50% aseptic glucose (6 mL/kg, intraperitoneally) to induce hyperglycemia 15 min before surgery.

Animals (n = 43) were prospectively excluded for the follow-up detection and analysis if they: 1) suffered subarachnoid hemorrhage (SAH) caused by the artery rupture after filament insertion; 2) died during the surgery caused by excessive gas anesthesia or excessive blood loss; 3) had a prolonged total operation time (>30 min); 4) were free of neurological deficits like forelimb hemiparesis while held by the tail and circling toward the paretic side; 5) survived less than 24 h.

### *Experimental groups*

Before the operation, animals were assigned to different groups: (1) Sham (n = 26); (2) Vehicle (n = 27); (3) Iron overload (n = 27); (4) Iron chelator (n = 27). 0.5 g/kg iron dextran (Sigma, USA) was injected intraperitoneally 6 h before MCAO to induce the status of acute intracranial iron overload as previous(Piloni et al., 2013). Once the filament was removed, the iron chelator group was intramuscularly administrated with the same dose of DFX (200 mg/kg, Sigma, USA) as other studies(van der Kooij et al., 2009; Won et al., 2011). Animals in the Vehicle and Sham groups were injected with equivalent 0.9% saline solution.

### *Mortality and neurological deficit score*

The mortality of animals was calculated and compared among groups. At 24 h after MCAO or Sham treatment, Bederson score (Bederson et al., 1986) and modified neurological severity score (mNSS) (Zarruk et al., 2011) were performed to test the neurological deficit of the animals. The Bederson score focused on movement tests with the maximum score of five. The mNSS mainly includes several aspects: 1) motor tests (muscle status-hemiplegia) (normal = 0; maximum = 6); 2) sensory tests (normal = 0; maximum = 2); 3) beam balance tests (normal = 0; maximum = 6); 4) reflexes absent and abnormal movements (normal = 0; maximum = 4). The total score of mNSS ranges from 0 to 18. All behavior measurements were carried out by an experienced researcher who was blinded to animal allocation.

### *Evaluation, classification and quantification of HT by spectrophotometric assay*

At 24 h after surgery, rats were transcardially perfused with 0.1 mol/L cold phosphate buffer saline (PBS) until the outflow fluid from the right atrium was clear. After that, their brains were removed and coronally incised. Intracerebral bleeding was evaluated by gross and microscopic observation: 1) Hemorrhage was observed by the naked eyes and coronal slices and bottoms of the brains were photographed; 2) Some brains were fixated with 4% paraformaldehyde and stained by hematoxylin–eosin (HE) to roughly assess the hemorrhage with a 20x objective lens. The incidence of HT and the type of hemorrhage was determined as NH, HI-1, HI-2 and PH following previously established criteria(Henninger et al., 2009) (n = 21 for each group).

HT was quantified using a spectrophotometric assay of brain hemoglobin content (QuantiChromTM Hemoglobin Assay Kit, BioAssay Systems, USA). After transcardiac perfusion with PBS, the right hemisphere was dissected out, homogenized, and centrifuged at 13,000g for 30 min. Supernatant (50 μl) from each sample was aliquoted into a 96 well plate and 200 μl of assay reagent was added. 50 μl water (Blank) and 50 μl Calibrator were pipetted into wells with 200 μl water. After 15 min incubation, optical density (OD) was measured at 400 nm with a Hybrid Multi-Mode Reader (Synergy H1, BioTek, USA). The total hemoglobin content of the infarct hemisphere was calculated as milligrams using a formula as $(OD_{sample} - OD_{Blank})/(OD_{Calibrator} - OD_{Blank})*Volume_{sample}$. Six rats were randomly selected from each group.

### *Extravasation of Evans blue*

At 22 h after operation, rats (n = 6 for each group) were intravenously injected with 2 mL/kg 2% Evans blue dye (Sigma, USA) 2 h before decapitation. Later they were transcardially perfused with 0.1 mol/L cold PBS. Brains were removed and the right hemispheres was weighed. The samples were placed in a 50% trichloroacetic acid solution and homogenized and centrifuged (10000 rpm for 20 min). The supernatant was diluted fourfold with ethanol. The fluorescence intensity was measured with the excitation wavelength at 620 nm and the emission wavelength of 680 nm by using a Hybrid Multi-Mode Reader (Synergy H1, BioTek, USA). The tissue content of Evans blue was quantified from a linear standard curve and was expressed as microgram per gram (μg/g) of brain tissue.

### *Immunohistochemistry*

At 24 h after MCAO or Sham treatment, rats were anesthetized by intraperitoneal injection of chloral hydrate. After transcardiac perfusion with 0.1 mol/L cold PBS and 4% paraformaldehyde in sequence, brains (n = 6 for each group) were isolated, fixed in 4% paraformaldehyde overnight at 4 ℃. 2 mm-thick coronal tissues within 1.2 mm anterior to 0.8 mm posterior from bregma were paraffin embedded and cut into 2 μm-thick sections involving the ipsilateral striatum and cortex. After routinely dewaxing, dehydration and antigen-repairing, sections were incubated overnight at 4 °C with anti-Ferritin Heavy Chain polyclonal antibody (1:100; PA5–27500, Invitrogen, USA), anti-Ferritin light chain polyclonal antibody (1:100; 10727–1-AP, Proteintech, China), anti-MMP9 polyclonal antibody (1:100; ab38898, Abcam, USA), anti-Occludin polyclonal antibody (1:400; PA5–30230, Invitrogen, USA), anti-ZO-1 polyclonal antibody (1:50; 61–7300, Invitrogen, USA) and anti-Claudin 5 monoclonal antibody (1:50; 35–2500, Invitrogen, USA)

followed by secondary antibody for 60 min at room temperature.

The tissue sections were observed by light microscopy and the fields of the ipsilateral cortex, striatum and peri-infarct area in each brain section were randomly chosen and photographed with a 40x objective lens. For counting immune-positive cells or quantifying integrated intensity, 10 randomly selected non-overlapping microscopic fields in each area were analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA). Five to six animals were picked in each group.

*Western blot analysis*

At 24 h after MCAO or Sham treatment, we dissected the right hemisphere of rats (n = 9 for each group). Extracts of brain tissues were prepared by homogenizing the tissue at 4 °C automatic sample grinder for 30 min in RIPA lysis buffer containing protease inhibitor cocktail. Extracts were centrifuged at 12000 rpm at 4 °C for 10 min. Supernatants were mixed with a 5X loading buffer containing dithiothreitol, and the mixtures were heated at 100 °C for 10 min. The mixtures were then subjected to SDS–polyacrylamide gel electrophores, after which proteins were transferred onto polyvinylidene fluoride membranes (Pall, USA). To detect ferritin, MMP-9, Occludin, ZO-1, and Cluadin-5, membranes were incubated at 4 ℃ overnight in 5% BSA dilutions of the following antibodies: anti-Ferritin Heavy Chain polyclonal antibody (1:1000; PA5–27500, Invitrogen, USA), anti-Ferritin light chain polyclonal antibody (1:500; 10727–1-AP, Proteintech, China), anti-MMP9 polyclonal antibody (1:1000; ab38898, Abcam, USA), anti-Occludin monoclonal antibody (1:1000; ab167161, Abcam, USA), anti-ZO-1 polyclonal antibody (1:250; 61–7300, Invitrogen, USA) and anti-Claudin 5 monoclonal antibody (1:2000; ABT45, Millipore, USA). Then membranes were incubated with the appropriate HRP-conjugated secondary antibody for 1 h at 37 °C. The blotted protein bands were visualized by enhanced chemiluminescence (Fusion Solo, Vilber, France) and quantified using Image J software (U.S. National Institutes of Health).

*Statistical analysis*

All data in this study were reported as mean±SD or count (percentage), and analyzed using SPSS software (version 23.0; IBM, Chicago, IL, USA). Inter-group differences were analyzed for statistical significance using one-way ANOVA followed by LSD post hoc analysis or multiple comparison of chi-square test after modifying test level. The distribution difference of hemorrhage type was analyzed by chi-square test or Fisher exact test. A threshold of two-side P value< 0.05 was identified as statistically significant.

## Results

A total of 150 rats had surgery performed. After exclusion of 43 animals (12 suffered SAH; 4 died during the surgery; 7 had a prolonged total operation time; 2 were free of neurological deficits; 18 survived less than 24 h), we included 107 animals in the analysis.

*Acute iron overload affects mortality and neurologic deficits in MCAO rats*

Five rats in the Vehicle group died within 24 h after surgery while no death occurred in the Sham group (15.6% vs 0.0%, P = 0.056, Fig. 1a). The Iron overload group had a higher mortality compared to the Vehicle group (30.8% vs 15.6%, P = 0.137, Fig. 1a). The neurological deficit score was higher in Vehicle animals than in Sham animals based on the Bederson score and mNSS (all P < 0.001, Fig. 1b). The Iron overload group had a more severe neurological deficits at 24 h after cerebral ischemia than the Vehicle group (2.63 ± 0.77 vs 1.92 ± 0.83 for Bederson score, P < 0.001; 11.96 ± 1.88 vs 9.67 ± 2.24 for mNSS, P < 0.001, Fig. 1b).

*Acute iron overload increases the incidence and severity of HT in rat brains*

Photographs of the brain and HE staining showed the difference of hemorrhage among groups (Fig. 2a). Compared with the Vehicle group, the Iron overload group had a higher proportion of PH and HI-2 (PH: 52.4% vs 4.8%; HI-2:42.9% vs 38.1%, all P < 0.05, Fig. 2c). A higher incidence of HT was found in the Iron overload animals compared to the Vehicle animals (100% vs 85.7%, P = 0.232, Fig. 2d). A significant difference of hemorrhage amounts was found in the Vehicle group compared with the Sham group (0.287 ± 0.138 mg vs 0.083 ± 0.012 mg, P = 0.001, Fig. 2b). Acute iron overload had a higher level of hemoglobin than the Vehicle group (0.446 ± 0.107 mg vs 0.287 ± 0.138 mg, P = 0.005, Fig. 2b).

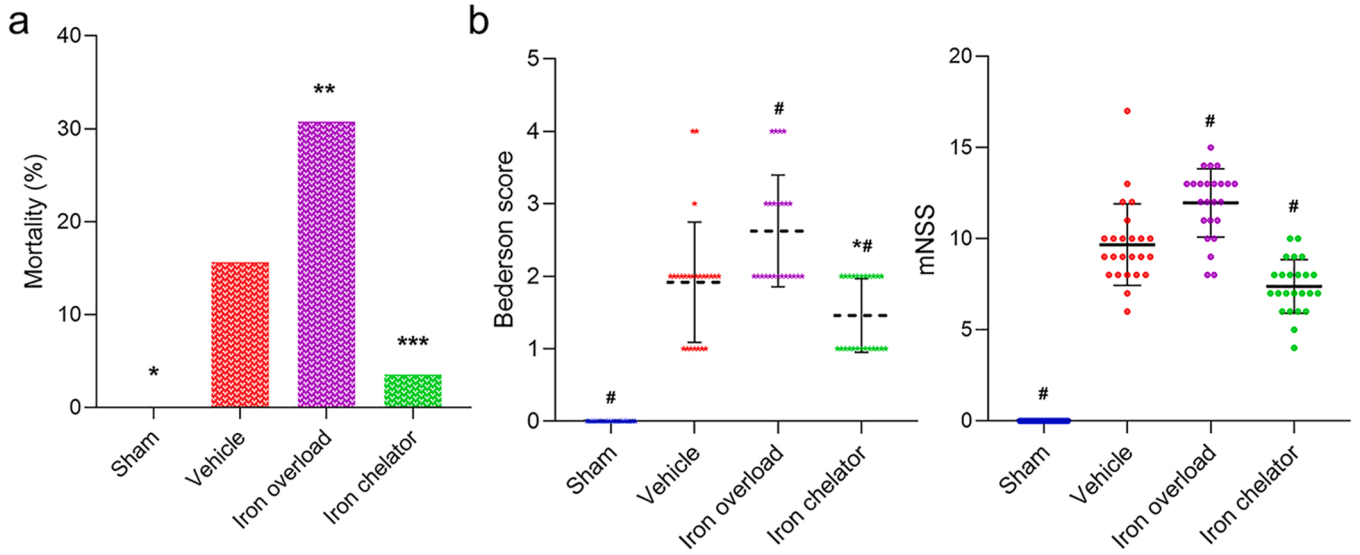


**Fig. 1.** Evaluation of mortality and neurological deficits among groups at 24 h after MCAO or Sham treatment. (a) Differences of mortality. *P = 0.056, vs Vehicle; * *P = 0.137, vs Vehicle; * **P = 0.201, vs Vehicle. (b) Bederson score and modified neurological severity score (mNSS) to determine the neurological function deterioration. (n = 24 per group). #P < 0.001 vs Vehicle. * #P = 0.012 vs Vehicle.

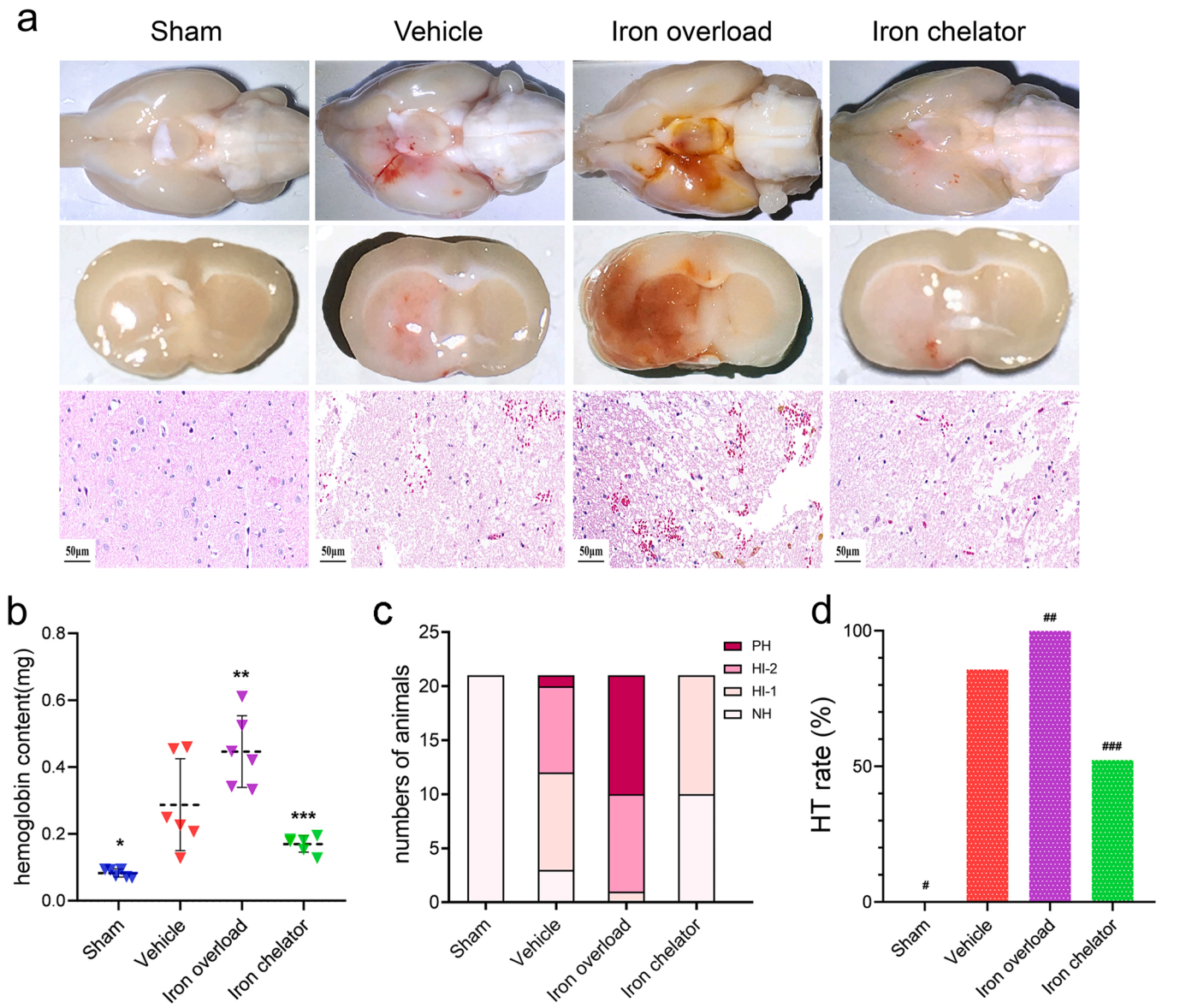


**Fig. 2.** Qualitative and quantitative analysis of brain hemorrhage after surgery. (a) Representative photographs from coronal slices and bottom of the brains (gross observation) and HE staining of brain sections (microscopic observation) in each group. (b) Brain hemoglobin content quantified by spectrophotometric assay among groups (n = 6 per group). *P = 0.001, vs Vehicle; * *P = 0.005, vs Vehicle; * **P = 0.032, vs Vehicle. (c) Differences stratified by hemorrhage type among groups (n = 21 per group). P < 0.001. (d) The incidence of hemorrhagic transformation (HT) in each group (n = 21 per group). #P < 0.001, vs Vehicle; ##P = 0.232, vs Vehicle; ###P = 0.019, vs Vehicle.

*Acute iron overload exacerbates BBB damage in MCAO rats*

Compared to the Sham group, the content of Evans blue extravasation in the right hemisphere was significantly increased in the Vehicle group (28.47 ± 5.93 vs 0.60 ± 1.47 μg/g, P < 0.001, Fig. 3). Acute iron overload further exacerbated the extravasation of Evans blue (61.86 ± 5.05 vs 28.47 ± 5.93 μg/g, P < 0.001, Fig. 3). By immunohistochemistry and western blot analysis, we found that levels of ZO-1, Occludin and Claudin-5 were significantly lower in the Vehicle group than the Sham group. Acute iron overload aggravated MCAO-induced reduction of levels of these TJs (all P < 0.05, Fig. 4a and 4b).

MMP-9 expression levels in the fields of the cortex, striatum and peri-infarct area were higher after MCAO than the Sham group by immunohistochemistry assay. Acute iron overload further enhanced this MCAO-induced increasing of MMP-9 level (all P < 0.05, Fig. 5a). The same inter-group differences of MMP-9 levels were observed by western blot analysis (all P < 0.05, Fig. 5b).

*Acute iron overload upregulates the expression of intracerebral ferritin in MCAO rats*

Immunohistochemistry showed a higher proportion of $FTH^+$ and $FTL^+$ cells in the fields of the cortex, striatum and peri-infarct area in the Vehicle animals compared to the Sham animals at 24 h after surgery. Compared to the Vehicle group, the Iron overload group had a significant higher proportion of $FTH^+$ and $FTL^+$ cells (all P < 0.05, Fig. 6a). Western blot analysis also showed increased levels of FTH and FTL in the Iron overload group compared with the Vehicle group (all P < 0.05, Fig. 6b).

*Iron chelator reverses pathologic impacts induced by iron overload in MCAO rats*

Administering DFX at 2 h after MCAO decreased mortality (15.6% vs 3.6%, P = 0.574, Fig. 1a) and the neurological deficit score (1.46 ± 0.51

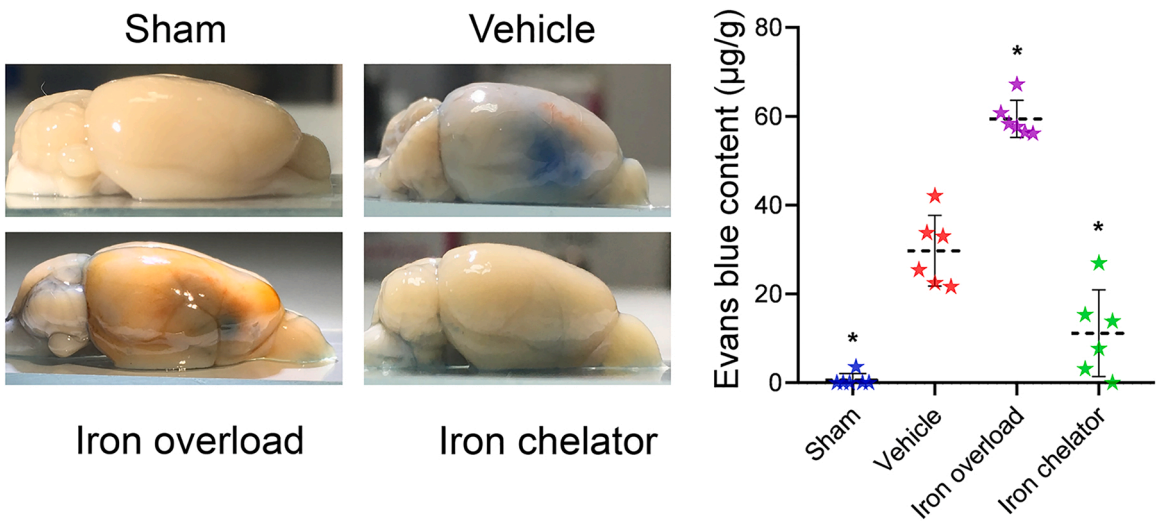


**Fig. 3.** Evaluation of blood-brain barrier (BBB) permeability in Sham, Vehicle, Iron overload and Iron chelator group at 24 h after MCAO or Sham treatment. Representative brain images of Evans blue extravasation in each group are shown. Content of Evans blue dye extravasation in the right hemisphere was measured and compared (n = 6 for each group). *P < 0.001, vs Vehicle.

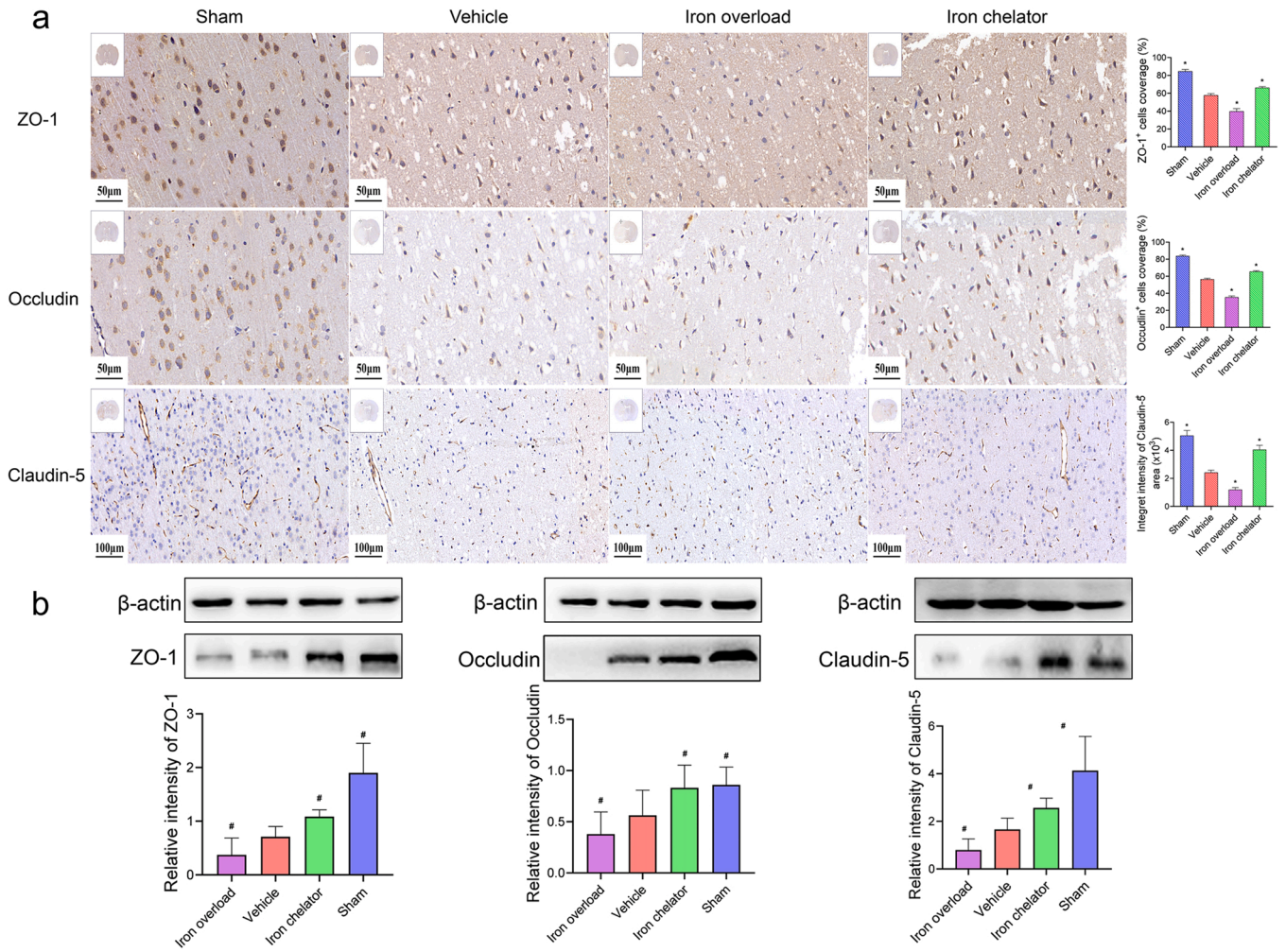


**Fig. 4.** Detection of tight junctions (TJs) expression levels in Sham, Vehicle, Iron overload and Iron chelator group after MCAO or Sham treatment. (a) Representative immunohistochemistry images of TJs (ZO-1, Occludin and Claudin-5). The slide overview and identified photographic location were shown in the upper-left corner of each image. The coverages of ZO-1$^{+}$ and Occludin$^{+}$ cells and integrated intensity of Claudin-5 positive expression area were shown in the panel below (n = 5–6 for each group). *P < 0.001, vs Vehicle. (b) Western blotting of tight junctions (TJs) in rat right infarct area. Representative images are shown in the upper panels and quantification of TJs in the lower panels are shown as mean±SD values for band relative intensity normalized to the intensity of β-actin. (n = 9 for each group). #P < 0.05, vs Vehicle.

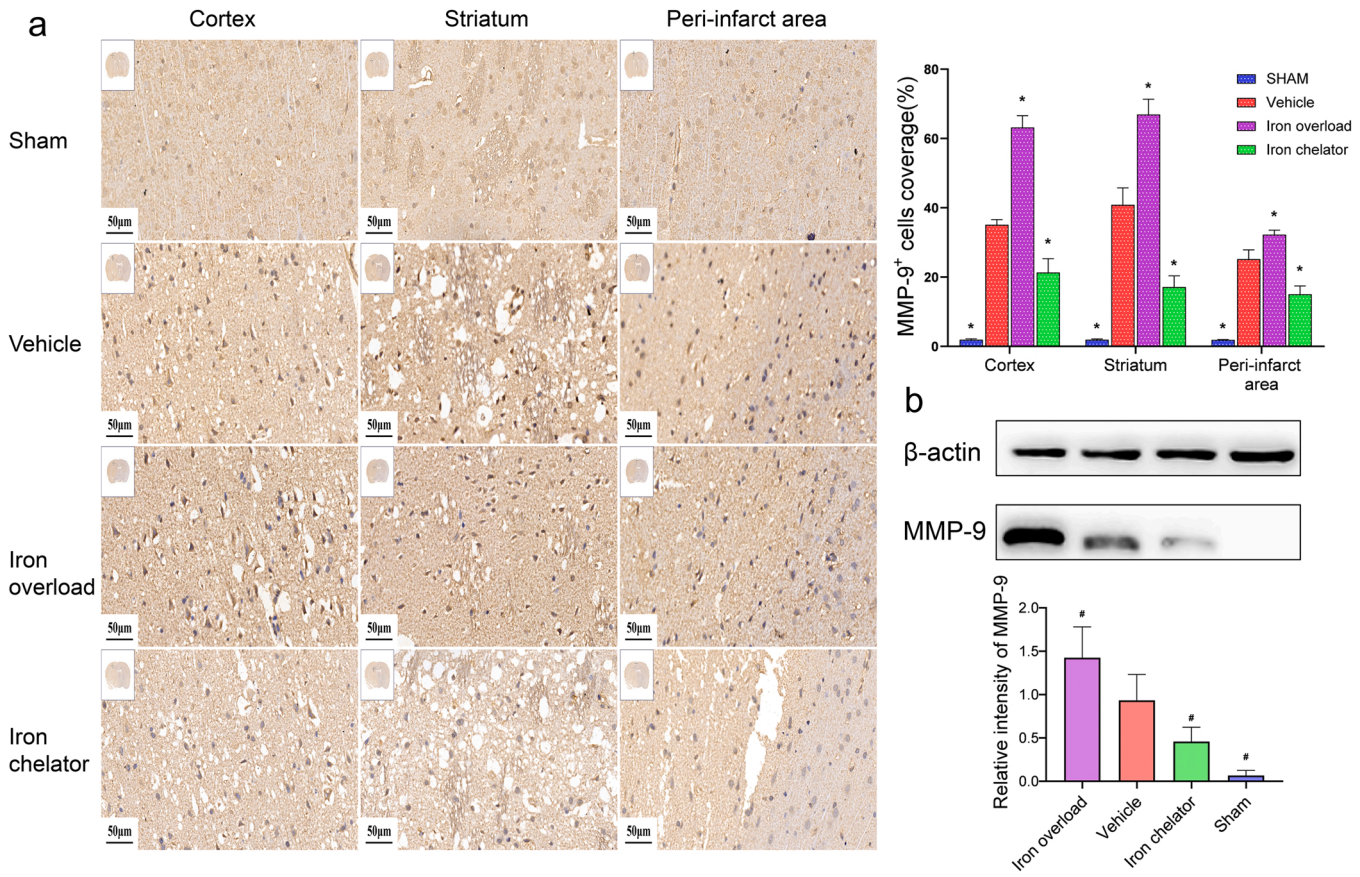


**Fig. 5.** Evaluation of metalloproteinase-9 (MMP-9) levels among groups after MCAO or Sham treatment. (a) Representative immunohistochemistry images of MMP-9. The slide overview and identified photographic location were shown in the upper-left corner of each picture. The coverages of MMP-9 $^{+}$cells counted in fields of ipsilateral cortex, striatum and peri-infarct area were shown respectively in the upper-right corner (n = 5–6 for each group). *P < 0.001, vs Vehicle. (b) Western blotting of MMP-9 in rat right infarct area. Representative images are shown in the upper panel and quantification in the lower panel are shown as mean±SD values for band relative intensity normalized to the intensity of β-actin. (n = 9 for each group). *P < 0.05, vs Vehicle.

vs 1.92 ± 0.83 for Bederson score, P = 0.012; 7.38 ± 1.47 vs 9.67 ± 2.24 for mNSS, P < 0.001. Fig. 1b) compared to the Vehicle group. We found animals treated with the iron chelator had significantly lower proportions of PH and HI-2 (PH: 0.0% vs 4.8%; HI-2: 0.0% vs 38.1%; all P < 0.05, Fig. 2c) and a lower incidence of HT (52.4% vs 85.7%, P = 0.019, Fig. 2d) than the Vehicle animals. Treatment with the iron chelator also had a significant effect of attenuating the extent of hemorrhage (0.287 ± 0.138 mg vs 0.170 ± 0.025 mg, P = 0.032, Fig. 2b). Animals administered with the iron chelator had a lower Evans blue extravasation (11.16 ± 9.76 vs 28.47 ± 5.93 μg/g, P<0.001, Fig. 3) in the right hemisphere than the Vehicle animals. Furthermore, immunohistochemistry and western blot analysis showed DFX significantly reversed the MCAO-induced reduction of TJs levels (all P < 0.05, Fig. 4a and Fig. 4b). Results from the immunohistochemistry and western blot analysis showed that administering DFX significantly reduced the MCAO-induced MMP-9 increasing (all P < 0.05, Fig. 5a and 5b). The intracerebral levels of FTH and FTL were decreased in animals injected with DFX (all P < 0.05, Fig. 6a and Fig. 6b).

## Discussion

The main results of our study were: 1) acute iron overload exacerbated BBB damage and HT after focal ischemic stroke in hyperglycemic rats; 2) the iron chelator significantly improved the iron overload status and ameliorated ischemia-reperfusion injury in rats with hyperglycemia.

A previous experimental study has verified iron overload induced by a nine-week high iron diet exacerbates the risk of tissue plasminogen activator (tPA)-induced HT in a thromboembolic model(Garcia-Yebenes et al., 2018), which is consistent with a clinical finding(Millan et al., 2007). Considering a very low rate of intravenous thrombolysis (2.5%–8.1%) in acute ischemic stroke(Wangqin et al., 2018), a great majority of ischemic stroke patients without tPA therapy should be also concerned about(Li et al., 2020). Previous studies have reported that over 1/3 of all ischemic stroke patients are accompanied by hyperglycemia, apparently increasing the risk of HT(Couret et al., 2018; Zang et al., 2021). Therefore, we provided complementary experimental evidence that iron overload aggravated HT in a hyperglycemia-MCAO rat model. In our study, acute intracerebral iron overload apparently increased the proportion of PH despite no tPA administration. Hence, whether iron overload increases the risk of HT in ischemic stroke patients accompanied with hyperglycemia should be investigated in further clinical studies.

Under normal physiological conditions, brain iron stored in the forms of ferritin mostly distributes in striatum, cortex and hippocampus. Pathological conditions of oxidative stress, inflammation and acidosis caused by cerebral ischemia-reperfusion may weaken the binding capacity of ferritin(DeGregorio-Rocasolano et al., 2019). Excess iron released from ferritin then binds to iron regulatory protein (IRP) machinery, which decreases the iron response element (IRE)'s affinity to ferritin mRNA and enhances the transcription of ferritin(Almutairi et al., 2019; DeGregorio-Rocasolano et al., 2019; Selim and Ratan, 2004; Shi et al., 2021). Increased expression of FTH and FTL have been found in cortex, striatum and peri-infarct area after experimental stroke in our study, indicating the existing iron overload status in the acute phase.

We also observed reduced intracerebral ferritin level by

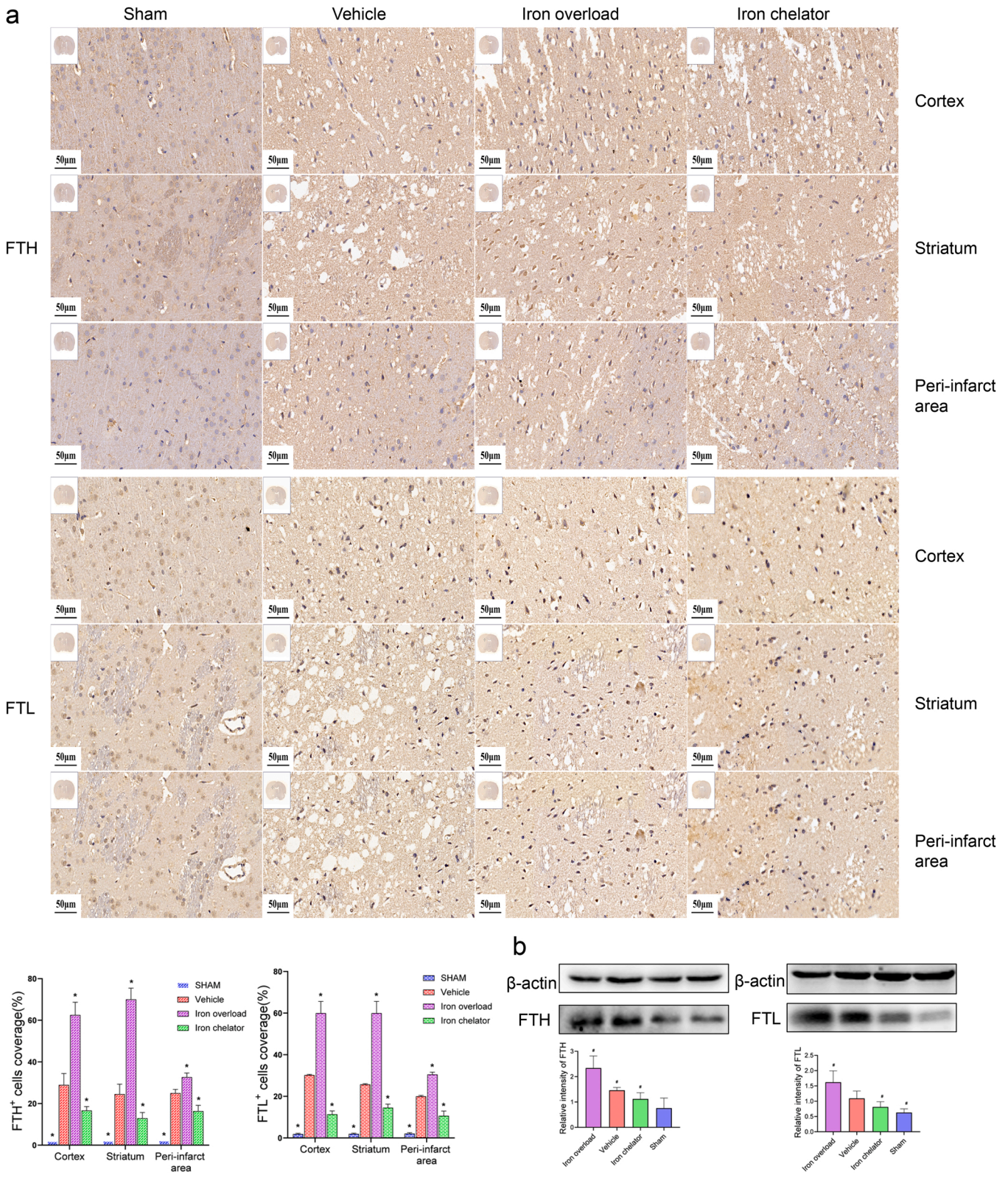


**Fig. 6.** Detection of ferritin expression in Sham, Vehicle, Iron overload and Iron chelator group after MCAO or Sham treatment. (a) Representative immunohistochemistry images of ferritin heavy chain (FTH) and ferritin light chain (FTL). The slide overview and identified photographic location were shown in the upper-left corner of each section. The coverages of FTH+ and FTL+ cells counted in fields of ipsilateral cortex, striatum and peri-infarct area were shown respectively in the lower-left corner (n = 5–6 for each group). All $P < 0.05$. (b) Western blotting of FTH and FTL in rat right infarct area. Representative images are shown in the upper panels and quantification in the lower panels are shown as mean±SD values for band relative intensity normalized to the intensity of β-actin. (n = 9 for each group). *$P < 0.05$, Vehicle vs Sham, Iron overload and Iron chelator respectively.

administering the iron chelator. Iron chelators have been explored for their therapeutic potential on experimental ischemic(Abdul et al., 2021; Edgerton et al., 2022) and hemorrhagic stroke(Caliaperumal et al., 2013; Imai et al., 2021). Deferoxamine, one of the most common iron chelators, has been reported to lessen the infarct volume, improve neurological deficits, reduce HT-induced injury and alleviate poor prognosis(Abdul et al., 2021; Almutairi et al., 2019; Shi et al., 2021) but not improve cognitive recovery in post-stroke animals(Edgerton et al., 2022). The roles of DFX in preventing BBB damage and HT complication, however, are reported in few studies. Xing et al. (2009) have found DFX attenuates HT mainly by accelerating the clearance of red blood cells and hemoglobin. Supplementarily, we found DFX mitigated HT by reducing iron overload and protecting BBB components against disruption after ischemic stroke. Thus, translational researches in effectiveness of this potential neuroprotective drug on preventing HT are further needed, based on its safety and trend to efficacy on improving outcomes in randomized clinical trials involving ischemic stroke patients(Millan et al., 2021).

Disruption of the BBB have been confirmed as the major pathophysiological mechanism of HT after ischemic stroke. Under normal condition, BBB acts as a selectable and dynamic barrier to protect the brain from unwanted compounds and maintain the homeostasis, depending on its special structural components including endothelial cells, junctional complex, basal membrane as well as other neurological cells(Bernardo-Castro et al., 2020). MMP-9, a classic matrix-degrading enzyme, has been widely studied in cerebrovascular diseases for its effects of degrading TJs and destroying basal membrane(Bauer et al., 2010). Our previous studies showed that MMP-9 is a key factor associated with HT in acute ischemic stroke patients(Yuan et al., 2018; Zheng et al., 2019). The findings of this study preliminarily indicated acute iron overload could increase MMP-9 levels, which may be one of the underlying mechanisms of aggravating BBB injuries and HT after ischemic stroke. Subsequent studies are needed to explore concrete molecular modulation mechanism.

At present, some studies have brought insights into the synergism of iron overload and hyperglycemia on the process of BBB damage. In cultured cells with high glucose, higher iron status could activate nuclear factor-κB pathway(Wu et al., 2020), which could upregulate MMP-9 expression(Liu et al., 2022). Excessive iron contributed to brain microvascular endothelial cells dysfunction in diabetic animals by increasing markers of ferroptosis and lipid reactive oxygen species (Abdul et al., 2021). Similarly in hyperglycemic patients with the most compensative mechanisms impaired, the organs or tissues are more susceptible to iron overload-induced oxidative stress(Liu et al., 2009). Chelating excessive iron attenuates brain injury in a rat model of cerebral ischemia with hyperglycemia(Xing et al., 2009) and improves functional outcomes in diabetic rats(Abdul et al., 2021).

The current study has several limitations which need to be addressed. First, HT may occur in a small part of patients without hyperglycemia or not undergoing thrombolytic therapy as a part of the natural history of cerebral infarction. Whether iron overload can increase the risk of HT in such groups is still to be determined. Second, this study only involved young, male SD rats. According to the recommendation of STAIR(Fisher et al., 2009), this study should be further performed in females, aged rats and animals with comorbidity conditions such as hypertension and hypercholesterolemia.

## Conclusion

Our results suggest that acute iron overload aggravates BBB damage and HT after transient ischemia in rats with hyperglycemia, which provides basic evidence for iron overload as a potential factor associated with BBB damage and HT in ischemic stroke patients when accompanied with hyperglycemia.

## Funding

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## Ethical Approval statement

The animal study protocol was evaluated and approved by the Animal Ethnics Committee, West China Hospital, Sichuan University (Approval No.2020257A).

## CRediT authorship contribution statement

M.L. and B.W. concepted and designed this study. Q.W., C.W. and S. G. carried out the animal experiment procedures and recorded the data. Q.W. and J.L. contributed to data analysis from animal experiments and interpretation. H.X. made provision for experimental materials and equipment. S.W. gave the administrative support. All authors wrote and approved the final manuscript.

## Conflicts of Interest

None.

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