Intracerebral Hematoma Contributes to Hydrocephalus After Intraventricular Hemorrhage via Aggravating Iron Accumulation

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Background and Purpose—The intraventricular hemorrhage (IVH) secondary to intracerebral hemorrhage (ICH) was reported to be relevant to a higher incidence of hydrocephalus, which would result in poorer outcomes for patients with ICH. However, the mechanisms responsible for this relationship remain poorly characterized. Thus, this study was designed to further explore the development and progression of hydrocephalus after secondary IVH.

Methods—Autologous blood injection model was induced to mimic ICH with ventricular extension (ICH/IVH) or primary IVH in Sprague-Dawley rats. Magnetic resonance imaging, Morris water maze, brain water content, Evans blue extravasation, immunohistochemistry staining, Western blot, iron determination, and electron microscopy were used in these rats. Then, deferoxamine treatment was used to clarify the involvement of iron in the development of hydrocephalus.

Results—Despite the injection of equivalent blood volumes, ICH/IVH resulted in more significant ventricular dilation, ependymal cilia damage, and iron overload, as well as more severe early brain injury and neurological deficits compared with IVH alone. Systemic deferoxamine treatment more effectively reduced ventricular enlargement in ICH/IVH compared with primary IVH.

Conclusions—Our results show that ICH/IVH caused more significant chronic hydrocephalus and iron accumulation than primary IVH alone. Intracerebral hematoma plays a vital role in persistent iron overload and aggravated hydrocephalus after ICH/IVH. (Stroke. 2015;46:00-00. DOI: 10.1161/STROKEAHA.115.009713.)

Key Words: brain injuries • cerebral hemorrhage • cerebrospinal fluid • hydrocephalus • iron

Intraventricular hemorrhage (IVH) occurs in 40% of patients with intracerebral hemorrhage (ICH). IVH is a severe complication of ICH and is rarely (<3%) found in the absence of ICH. Recent studies have shown that IVH and hydrocephalus are predictors of poor outcome after ICH. Both human studies and preclinical studies on primary IVH or ICH with ventricular extension (ICH/IVH) are rare. However, some clinical studies have indicated that IVH, secondary to ICH, could lead to a higher risk of longterm shuntdependent hydrocephalus compared with primary IVH. Brain injury and hydrocephalus have not been systematically evaluated in relation to these 2 types of IVH in either humans or animals. Recently, based on the most commonly used primary IVH rat model, we established a rat model of reproducible ICH/IVH, which features characteristics of both ICH and IVH and closely mimics human IVH.

Although it is well known that IVH is associated with hydrocephalus, the underlying mechanisms remain unclear. Recent studies have indicated that iron may play an important role in posthemorrhagic hydrocephalus and brain injury.

After disruption of the brain–cerebrospinal fluid (CSF) barrier because of IVH, intracerebral hematoma may provide a source of iron for release into the ventricular system via this breached barrier, eventually leading to brain damage.

Thus, we hypothesized that ICH/IVH would generate more significant hydrocephalus and brain injury than IVH alone. This study examined this hypothesis by comparing the ICH/IVH rat model with the primary IVH rat model. Moreover, we explored the potential role of intracerebral hematoma in brain iron deposition and hydrocephalus after ICH/IVH.

Methods

One hundred and sixty adult male Sprague-Dawley rats (250–350 g; the Third Military Medical University) were used. Animal use procedures were in compliance with the Guide for the Care and Use of Laboratory Animals and approved by the Animal Care and Use Committee of Laboratory Animals and approved by the Animal Care and Use Committee of Laboratory Animals and approved by the Animal Care and Use Committee of Laboratory Animals and approved by the Animal Care and Use Committee of Laboratory Animals and approved by the Animal Care and Use Committee of Laboratory Animals and approved by the Animal Care and Use Committee of Laboratory Animals and approved by the Animal Care and Use Committee of Laboratory Animals and approved by the Animal Care and Use Committee of Laboratory Animals and approved by the Animal Care and Use Committee of Laboratory Animals and approved by the Animal Care and Use Committee of Laboratory Animals.
Committee at the Third Military Medical University. Animals were anesthetized with pentobarbital (40 mg/kg IP), and the right femoral artery was catheterized to monitor arterial blood pressure, blood pH, PaO₂, PaCO₂, and glucose levels (Table I in the online-only Data Supplement). A feedback-controlled heating pad was used to maintain body temperature at 37.0°C. A cranial burr hole (1 mm) was drilled, and a 29-gauge needle was inserted stereotaxically into the right lateral ventricle (coordinates: 0.6 mm posterior, 4.5 mm ventral, and 1.6 mm lateral to the bregma) to establish the ICH model (Figure I in the online-only Data Supplement). For the model of ICH with ventricle extension, the needle was inserted stereotaxically into the right caudate nucleus (coordinates: 0.2 mm posterior, 5.0 mm ventral, and 2.2 mm lateral to the bregma; Figure I in the online-only Data Supplement). Autologous arterial blood was infused at a rate of 14 μL/min using a microinjection pump. The burr hole was sealed with bone wax, and the skin incision was closed with sutures after the needle was removed. The sham groups required only needle injection into the right caudate nucleus as the ICH/IVH rats.

This study was divided into 4 parts (Figure II in the online-only Data Supplement). First, rats had a right lateral ventricular or intracaudate injection of 200 μL of autologous whole blood. The sham control received only needle injection. All rats were euthanized at 24 hours after infusion for brain water content (n=9 for each group). Evans blue extravasation determination (n=9 for each group), or continuous tissue slicing (n=3 for each group) after T2-weighted magnetic resonance imaging. Second, rats (n=11 for each group) received an injection of blood or sham operation as part 1 and then underwent serial magnetic resonance imaging scans and a CSF tap for iron determination at days 1, 3, 8, 14, and 28. Then, these rats were euthanized for immunohistochemistry, immunofluorescence, and electron microscope analysis, respectively. Third, rats had an injection of 200 μL of blood into right lateral ventricle or striatum. All rats were tested in the Morris water maze from days 23 to 28 after infusion. Then, the rats were euthanized at day 28 for brain total iron determination (n=7 for each group) and Western blot analysis (n=4 for each group). Fourth, rats received an injection of blood or sham operation as part 1 and had deferoxamine (100 mg/kg IM, n=8) or vehicle (same volume of saline, n=6) treatment at 2 and 6 hours after infusion and then every 12 hours for 7 days. The dose regimen of deferoxamine was referred to previous studies. Magnetic resonance imaging scans were conducted at days 1, 3, 8, 14, and 28.

The values in this study are presented as mean±SD. Data were analyzed by Student t test for single comparisons or ANOVA with post hoc Bonferroni−Dunn correction for multiple comparisons. A P value of <0.05 was considered statistically significant.

Results

ICH/IVH Induced More Severe Early Brain Injury Than IVH Alone

Twenty-four hours after blood infusion, the ICH/IVH rats showed both intracerebral hematoma and ventricular system hemorrhage (Figure 1A), whereas the IVH animals showed only ventricular system hemorrhage (Figure 1B). Despite similar initial blood volume (200 μL) injection, the brain water content of the ipsilateral hemisphere in the ICH/IVH group was much higher than that in the IVH group (79.74±0.39 versus 78.79±0.37; P<0.01; Figure 1C). Evans blue extravasation in the contralateral hemisphere in the ICH/IVH group was much higher than that in the IVH group (13.69±2.55 versus 5.10±1.11; P<0.01; Figure 1D); the same pattern was seen in the ipsilateral hemisphere (20.98±3.48 versus 9.19±2.13; P<0.01; Figure 1D). In addition, the Evans blue fluorescence also revealed a more significant perivascular Evans blue extravasation in ICH/IVH rats (Figure III in the online-only Data Supplement).

ICH/IVH Rats Developed More Significant Long-Term Ventricular Dilation

At 24 hours after blood injection, the lateral ventricular volumes in ICH/IVH rats were much smaller than those in IVH rats (41.52±8.62 versus 62.48±7.99 mm³; P<0.01; Figure 2A and 2B). However, at day 14, the ICH/IVH animals showed a significantly greater ventricular dilation than IVH animals (60.76±10.17 versus 42.21±5.77 mm³; P<0.01; Figure 2A and 2B), which peaked at day 28 (65.31±10.11 versus 41.73±5.82 mm³; P<0.01; Figure 2A and 2B).

ICH/IVH Aroused More Severe Ependymal Cilia Damage

Twenty-eight days after infusion, in ICH/IVH samples, ependymal cells had more serious ciliary defects, including shorter cilia, a reduced number of cilia per cell, or the absence of cilia (arrows), compared with the IVH group (Figure 3B). Electron micrographs showed the ultrastructure of ependymal cells of the ipsilateral right ventricle at day 28 after IVH or ICH/IVH. The scanning electron and transmission electron microscopy analyses revealed significant ependymal cilia loss after ICH/IVH, whereas IVH alone led to minor cilia injury (Figure 3A).

ICH/IVH Led to More Serious Hippocampus Injury

Twenty-eight days after infusion, hippocampal volumes were smaller in ICH/IVH rats compared with IVH rats (82.33±3.15 versus 86.69±2.62 mm³; P<0.05; Figure 4A and 4B in the online-only Data Supplement). There was also a significant reduction in neuron-positive cells in the CA1 area of the hippocampus in ICH/IVH rats (85±18 versus 114±23 mm³; P<0.05; Figure 4C and 4D in the online-only Data Supplement) at this time point. No mortality was found in either ICH/IVH or IVH rats despite neuronal death in the hippocampus.

ICH/IVH Caused More Severe Neurocognitive Functional Deficit

With 5 consecutive days (days 23–37) of acquisition training, there was a remarkable increase in the latencies to the goal in the ICH/IVH group compared with the IVH group (P<0.05 or 0.01; Figure VA in the online-only Data Supplement), indicating that ICH/IVH induced more severe learning deficits than did IVH. From the first day to sixth day, there was no difference in swimming speed between the ICH/IVH and IVH groups (P>0.05; Figure VB in the online-only Data Supplement), which, to some extent, excluded the interference of motor behavior on cognitive assessment from ICH. During the probe trial (day 28), the ICH/IVH groups performed fewer platform crossings than did IVH rats (P<0.01; Figure VC in the online-only Data Supplement). In addition, the ICH/IVH animals spent less time traveling in the target quadrant than did the IVH group (P<0.01; FigureVD in the online-only Data Supplement), further suggesting that ICH/IVH induced more severe memory deficits than did IVH.
As a Source of Iron, Intracerebral Hematoma Induced More Significant Brain Tissue/CSF Iron Accumulation After ICH/IVH

Enhanced Perls staining and graphite furnace atomic absorption spectrometry were used to examine iron accumulation, and more iron-positive cells were found in the ependyma and subependyma of ICH/IVH rats than IVH rats (Figure 4A). Total brain iron content in the ipsilateral hemisphere at day 28 (17.18±3.42 versus 8.69±2.93 µg/g; P<0.01; Figure 4B) was also much higher in the ICH/IVH group compared with the IVH group. The iron concentration of CSF at day 1 after IVH was higher than the ICH/IVH group; however, the ICH/IVH group reversed and exceeded IVH from day 3 to day 28 (day 14: 10.07±3.60 versus 6.95±11.94 µmol/L; P<0.01; Figure 4C). With equal initial blood injection, the ICH/IVH group caused more significant brain tissue/CSF iron accumulation than IVH alone, which further supported our hypotheses that intracerebral hematoma may be a source of iron after ICH/IVH.

ICH/IVH Caused More Remarkable Ferritin Expression in the Periventricular Zone

Ferritin levels were examined by Western blot and immunohistochemistry. Despite similar initial blood injections, ICH/IVH rats showed not only more ferritin-positive cells (210±49 versus 113±34; P<0.01; Figure 5A) but also much higher levels of both ferritin light chains and ferritin heavy chains in the periventricular zone compared with the IVH group after 28 days (Figure 5B and 5C).

Deferoxamine Better Alleviated Ventricular Size in ICH/IVH Than in IVH Alone

In agreement with our previous publication,12 deferoxamine treatment (100 mg/kg IM at 2 and 6 hours after IVH and then every 12 hours for 7 days) significantly reduced lateral ventricular dilation at 14 days (35.98±5.83 versus 46.04±6.01 mm³ in the vehicle-treated group; P<0.05) and 28 days (31.89±5.51 versus 41.10±6.83 mm³ in the vehicle-treated group; P<0.05; Figure 6A and 6B) after IVH, as well as after ICH/IVH (14 days: 42.52±6.97 versus 59.34±10.10 mm³ in the vehicle-treated group; P<0.05; Figure 6A and 6B). In addition, we found that deferoxamine treatment reduced the lateral ventricular volume at 28 days (17.98±5.12 versus 9.20±4.79 mm³ in IVH group; P<0.05; Figure 6A and 6C) after ICH/IVH in comparison with IVH.

Discussion

The major findings of this study are as follows: (1) despite the injection of equal amounts of blood, ICH/IVH rats showed more severe long-term lateral ventricular dilation and brain iron accumulation compared with rats subjected to IVH alone; (2) intracerebral hematoma contributed to iron overload and aggravated hydrocephalus after ICH/IVH in rats; and (3) deferoxamine, an iron chelator, better alleviated hydrocephalus in ICH/IVH rats than in IVH rats.

Little attention has been paid to preclinical studies or clinical trials that studied primary IVH or ICH/IVH. However, limited retrospective studies have suggested that ICH/IVH could lead to a higher risk of longterm shuntdependent hydrocephalus, compared with primary IVH.5–9 Among patients with primary IVH, a ventriculoperitoneal shunt was eventually placed in 2 of 29 (7%) patients, and ≈121 of 312 (38%) did not survive hospital discharge.5–7 After ICH/IVH, permanent ventricular CSF shunting was performed in 13 of 64 (20.3%) patients, and the actual mortality was as high as 679 of 1204 (56.36%).6,8 To our knowledge, this is the first
stroke to compare primary IVH with ICH/IVH. Unlike previous primary IVH rat models,12 the present ICH/IVH rat model features characteristics of both ICH and IVH, which more effectively mirror the human IVH pathology than previous IVH rat models. In agreement with our previous publications,11,12 we observed that ICH/IVH animals developed more significant ventricular expansion at 14 and 28 days after infusion compared with IVH rats. Brain atrophy and tissue loss after ICH could enhance ventricular size,17 which may interfere with the IVH-induced ventricular dilation. However, previous studies demonstrated that ICH-induced expansion was mainly located in perihematomal levels or the ipsilateral ventricle,17,18 whereas our ICH/IVH rats developed wide and equal bilateral ventricular expansion.11 Furthermore, this ICH/IVH model formed a much smaller hematoma than rat models of ICH alone. Therefore, we could exclude interference from ICH on ventricular dilation after IVH in this study.

There is increasing evidence that iron is involved in hydrocephalus after IVH.12,13,15,19–21 During IVH, a channel forms between the intracerebral hematoma and ventricles, and the intracerebral hematoma may serve as a source of iron for release into the ventricular system, eventually leading to brain damage. Therefore, we postulate that intracerebral hematoma–derived iron may play an important role in the reversed ventricular expansion curve after ICH/IVH (Figure 2). We investigated 2 types of IVH models and observed that ICH/IVH animals developed more significant iron overload and ferritin expression in the periventricular zone despite the same initial blood volume (200 μL) injection. Thus, our results indicated that intracerebral hematoma may be a source of iron after ICH/IVH, which gradually worsened ventricular expansion via the persistent iron release into the ventricles. Furthermore, deferoxamine treatment more effectively alleviated hydrocephalus at 28 days after ICH/IVH, further supporting our hypotheses. Future studies are needed to further clarify the underlying mechanisms. Moreover, clinical trials are also required to explore whether evacuation of hematoma in early stage is an effective way to prevent shunt-dependent hydrocephalus after ICH/IVH.

At present, the mechanisms associated with iron-mediated hydrocephalus remain incompletely understood. Traditional explanations generally involve inflammatory pathways and scarring of the CSF outflow.4 Recently, an increasing body of evidence has shown that iron-related hydrocephalus results from ependymal cell injury. Gao et al13 also found that iron may cause ependymal cell death and loss of cilia, suggesting the possibility that ependymal cell injury may result in
defective CSF dynamics and aggravated hydrocephalus. In addition, we previously reported ependymal surface damage and loss of cilia on the ventricular wall at 28 days after IVH. Moreover, treatment with edaravone, a free-radical scavenger, could attenuate hydrocephalus and cilia abnormality by decreasing iron-induced oxidative injury after IVH.

Figure 4. A. Perls reaction showed more iron-positive cells in the ependyma and subependyma in intracerebral hemorrhage (ICH)/intraventricular hemorrhage (IVH) compared with IVH rats at day 28 after infusion. Scale bar, 20 μm. Brain total iron at day 28 (B), where the values are expressed as mean±SD, n=7; **P<0.01 versus IVH group. Cerebrospinal fluid (CSF) iron determination at days 1, 3, 8, 14, and 28 (C), where the values are expressed as mean±SD, n=11; *P<0.05 versus IVH group.

Figure 5. A. Immunoreactivity of ferritin in the periventricular zone (PVZ) and ferritin (+) cell counts at day 28 after intraventricular hemorrhage (IVH) or intracerebral hemorrhage (ICH)/IVH. Values are expressed as mean±SD, n=6; **P<0.01 versus IVH group. Scale bar, 20 μm. Western blot analysis showing protein levels of ferritin-L-chain (B) and ferritin-H-chain (C) in the PVZ at day 28 after infusion. Values are expressed as mean±SD, n=4; **P<0.01 versus IVH group.
In this study, we observed that ICH/IVH led to much higher iron levels in the CSF and periventricular zone, as well as more severe ependymal cilia damage, compared with primary IVH (Figure 3). Our study thus suggests that iron-mediated ependymal cilia injury may result in defective CSF dynamics and aggravate posthemorrhagic hydrocephalus.

In addition to iron, many factors, such as transforming growth factor-β1, may also contribute to hydrocephalus after ICH/IVH. In 2004, Heep et al23 and Cherian et al 24 reported elevated transforming growth factor-β1 expression in the CSF of premature infants with posthemorrhagic hydrocephalus and periventricular area of rat model with posthemorrhagic hydrocephalus, respectively. In addition, we previously observed that transforming growth factor-β1 highly expressed in perihematomal tissue of neonate rat models of germinal matrix hemorrhage, and SD208, a potent inhibitor of transforming growth factor receptor-I, effectively ameliorated germinal matrix hemorrhage–induced ventriculomegaly. 25 But, because of limited evidences, further studies are still needed to explain this phenomenon. Apart from iron, many factors such as thrombin, inflammation, and intracerebral hematoma–mediated mass effect and clot retraction, may also play a role in this early edema. In addition, ICH/IVH rats also showed a more serious damage in learning and memory ability and hippocampal neurons than IVH alone. Whether ICH/IVH patients will have more severe neurocognitive disorders than patients with primary IVH requires further validation in clinical trials.

Conclusions

Our results showed that ICH combined with IVH in rats caused more significant long-term hydrocephalus than IVH alone, which well reproduced the clinical situation. Our data
further showed that intracerebral hematoma contributed to persistent brain iron accumulation and aggravated hydrocephalus after ICH/IVH. Moreover, this study provides evidence that deferoxamine may be a potential therapeutic for ICH patients with ventricular extension, especially on preventing chronic hydrocephalus.

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Disclosures
None.

References
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SUPPLEMENTAL MATERIAL

Brain water content

Twenty-four hours after blood infusion, rats were euthanized. Brains were removed and divided into two hemispheres and the cerebellum. The wet weights were determined immediately. Then, the brain sections were dried at 100°C for 24 hours before obtaining the dry weights. The percentage of water content was calculated as follows: (wet weight – dry weight)/wet weight × 100.

Vascular permeability measurement

Vascular permeability was determined by measuring the amount of Evans blue (EB) dye (Aladdin, USA) extravasation 24 hours after blood infusion. Rats were injected with 2% EB dye (5 ml/kg) via the tail vein 60 minutes before perfusion and brain removal, as previously described.¹ The content of EB was measured as ng/mg brain tissue using a standardized curve. For Evans blue fluorescence, the brains were removed to be prepared for coronal brain sections. And, red autofluorescence of Evans blue was observed on the slides as previously described.²

MRI and volume measurement

Rats were anesthetized with 2% isoflurane/air mixture throughout MRI examination. The MRI scans were performed in a 7.0-T Varian MR scanner (Bruker, USA) with a T2 fast spin-echo sequence using a view field of 35 mm × 35 mm and 17 coronal slices (1.0 mm thickness). Volumes were calculated as previously described.³ Bilateral ventricles and the hippocampus were outlined, and the areas were measured; volumes were assessed by calculating the areas of all slices and multiplying by the
section thickness. All image analyses were performed using Image J (National Institutes of Health, Bethesda, Maryland, USA) by two observers in blinded manner.

**Neurocognitive function assessment**

Twenty-three days after blood infusion, the Morris water maze test was performed to assess learning and memory of the animals, as previously described. Animals were placed in a metal pool (50 cm in depth, 200 cm in diameter) filled with water and were allowed to find the submerged platform within 120 s. Then, they were given 5 days of acquisition training using a random set of start locations in the four quadrants. The latency time was monitored and averaged across four trials per day. The swimming speed was recorded from first day to the sixth day. On the sixth day, each rat was subjected to a probe trial (120 seconds) in which the platform was removed. The platform crossing times in each quadrant and the percent time in the target quadrant were calculated.

**Cell counts**

The cell counts were performed at day 28 after infusion. For quantification of the positive cells in the periventricular area (-0.4mm posterior to bregma) and hippocampus area (-3.3mm posterior to bregma), consecutive slices were made, and 2 sections per animal (n=6 per group) with 40 μm space in between were used for cell counts. Three high-power images (40× magnification) per section were obtained as the black boxes (Fig 5A). Ferritin-positive cells were counted in the periventricular area. NeuN-positive cells were counted in the hippocampal area. Cell counts were performed by two researchers in a blinded manner. All measurements were repeated
three times, and the mean value was used.

**Enhanced Perls’ reaction**

Enhanced Perls’ reaction was performed as previously described. Brain sections were incubated in Perls’ solution (1:1, 5% potassium ferrocyanide and 5% HCl) for 45 minutes, washed in distilled water, and incubated again in 0.5% diamine benzidine tetrahydrochloride with nickel for 60 minutes.

**Total brain tissue iron and CSF iron determination**

CSF was obtained at days 1, 3, 8, 14, and 28 after infusion by puncture of the cisterna magna and stored at -80°C prior to analysis. The brains were perfused with saline before decapitation at day 28. Left and right hemispheres were sampled and weighed. Total brain tissue iron (μg/g tissue weight) and CSF iron (μmol/L) were determined using graphite furnace atomic absorption spectrometry after microwave-assisted acid digestion of the samples according to the method described by Ramos et al.

**Western blot analysis**

Western blot analysis was performed as previously described. The brains were perfused with saline before decapitation at day 28 after injection. The periventricular brain tissue (1-mm-thick brain tissue around the ventricle) was sampled. The primary antibodies included rabbit anti-ferritin heavy chain polyclonal antibody (1:2,000 dilution; Cell Signaling Technology) and goat anti-ferritin light chain poly-clonal antibody (1:2,000 dilution; Abnova). The relative densities of the bands were analyzed using NIH ImageJ software.

**Dissection of lateral ventricles and scanning electron microscopy**
Animals were decapitated under deep anesthesia, and the brains were removed immediately. The right lateral ventricles were exposed widely using the whole-mount dissection technique previously described. Brain samples containing the dorsal–lateral wall of the right lateral ventricle were then prepared for scanning electron microscopy (FEI Quanta 450; FEI Company, Hillsboro, Oregon, USA) as previously described. Briefly, the tissues were fixed in Sorensen’s phosphate-buffered glutaraldehyde (4%, pH 7.4) for 48 hours. After post-fixation in 1% osmium tetroxide, samples were dehydrated through ethanol gradients and dried. After sputter coating with platinum, the mounted samples were ready for examination.

**Transmission electron microscope**

Electron microscopy was performed as previously described. Rats were anesthetized and subjected to intracardiac perfusion with 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 mol/L Sorensen’s buffer (pH 7.4). The brains were removed and a 1-mm-thick coronal brain slice was cut with a blade 4 mm from the frontal pole. The ipsilateral ventricular wall tissue was sampled and immersed in the same fixative overnight at 4°C. Samples were then post-fixed with 1.0% OsO4 and dehydrated in graded ethyl alcohol. After completion of dehydration, samples were infiltrated with propylene oxide, embedded in Epon resin, and sectioned. Ultra-thin sections were then stained with uranyl acetate and Reynold’s lead citrate. Sections were evaluated using a Philips CM 100 transmission electron microscope (Hillsboro, OR, USA) and digitally acquired using a Hamamatsu (Hamamatsu City, Shizuoka, Japan) ORCA-HR camera.
Immunofluorescence and immunohistochemistry staining

Immunofluorescence staining of brain tissue was performed on fixed frozen sections as previously described. Rats were anesthetized with pentobarbital (100 mg/kg intraperitoneal) and perfused with 4% paraformaldehyde in 0.1 mol/L, pH 7.4 PBS. The brains were removed and kept in 4% paraformaldehyde for 4 to 6 hours and then immersed in 30% sucrose for 3 to 4 days at 4°C. The brains were embedded in an optimal cutting temperature compound (SAKURA, USA), and 18-mm-thick slices were cut using a cryostat. The following primary antibodies were used: mouse antibody to NeuN (1:1,000, Millipore), rabbit antibody to S100 (1:500, Sigma), and mouse monoclonal anti-α-tubulin-FITC antibody (1:500, Sigma). The secondary antibodies included Alexa Fluor 488-conjugated goat anti-rabbit mAb (1:800, Jackson), Alexa Fluor 488-conjugated goat anti-mouse mAb (1:800, Jackson), and Alexa Fluor 555-conjugated goat anti-mouse mAb (1:800, Jackson). Immunohistochemical studies were performed using the avidin–biotin complex technique as previously described, and the primary antibody was polyclonal rabbit anti-human ferritin IgG (1:400; DAKO).
**Supplemental Table 1.** Physiological parameters of rats before and after infusion. All parameters showed no significant differences between each groups during infusion.

<table>
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<th>ICH/IVH (n=4)</th>
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**Supplemental Figure I.** Schematic diagram of models injection.

**Primary IVH**
- 0.6mm posterior
- 1.6mm lateral
- 4.5mm ventral

**ICH+IVH**
- 0.2mm anterior
- 2.2mm lateral
- 5.0mm ventral
Supplemental Figure II. Experimental protocol. CSF indicates cerebrospinal fluid; IVH, intraventricular hemorrhage; ICH/IVH, intracerebral hemorrhage combined with IVH; DFO, deferoxamine.

**Part 1**
- Sham (n=21)
- IVH (n=21)
- ICH/IVH (n=21)

**Part 2**
- Sham (n=11)
- IVH (n=11)
- ICH/IVH (n=11)

**Part 3**
- IVH (n=11)
- ICH/IVH (n=11)

**Part 4**
- Sham+Vehicle (n=6)
- IVH+Vehicle (n=6)
- ICH/IVH+Vehicle (n=6)
- Sham+DFO (n=8)
- IVH+DFO (n=8)
- ICH/IVH+DFO (n=8)
**Supplemental Figure III**  Peri-vascular Evans blue fluorescence of ipsilateral cortex at 24h after infusion. The ICH/IVH group showed more significant Evans blue extravasation than both IVH and sham groups. The scale bars indicate 50μm.
Supplemental Figure IV. (A) Measurement of hippocampus volume with T2-weighted MRI 28 days after IVH or ICH/IVH. The hippocampus is outlined on the images. (B) The hippocampus volumes are expressed as the mean ± SD (n = 11). *P<0.05 vs IVH group. (C) Immunofluorescence of neurons in the CA1 zone of the hippocampus (D) The number of NeuN (+) are expressed as the mean ± SD *P<0.05 vs IVH group. Scale bar represents 20 μm.
**Supplemental Figure V.** Neurocognitive function assessment at days 23 to 28 after infusion. (A) Escape latency in training trials, (B) Swimming speed in training trials, (C) Platform crossing times in probe trials, (D) Percent time in the target quadrant in probe trials, (E) Representative swim traces in probe trials. Values are expressed as the means ± SD, Values are expressed as the mean ± SD, n = 11 per group; *P<0.05, **P<0.01 versus the IVH group.
Supplemental References


