Role of Iron in Brain Injury After Intraventricular Hemorrhage

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Background and Purpose—Intraventricular extension of hemorrhage is a predictor of poor outcome in intracerebral hemorrhage, and iron overload contributes to brain injury after intracerebral hemorrhage. The current study investigated the role of iron in ventricular dilatation and neuronal death in a rat model of intraventricular hemorrhage (IVH).

Methods—There were 2 parts in this study. First, male Sprague-Dawley rats had a 200-μL injection of either autologous blood or saline into the right lateral ventricle and were euthanized at different time points. Rats had MRI and brains were used for Western blot analysis, immunohistochemistry, histology, and brain tissue nonheme iron measurements. Second, rats had IVH and were treated with deferoxamine or vehicle, and rats were euthanized 4 weeks later for brain tissue loss and lateral ventricle size measurements.

Results—IVH resulted in brain iron accumulation, bilateral enlargement of the lateral ventricles, and hippocampal brain tissue loss. Iron accumulation was associated with upregulation of heme oxygenase-1 and ferritin. Systemic deferoxamine treatment reduced IVH-induced ventricular enlargement (eg, day 28: 32.7 ± 10.6 vs 43.8 ± 9.7 mm³ in vehicle-treated group, n = 8 to 9; P < 0.05) and hippocampal brain tissue loss (hippocampal volume: 89.0 ± 2.7 vs 85.2 ± 4.1 mm³ in the vehicle-treated group; P < 0.05).

Conclusions—Iron has a role in brain injury after IVH. Deferoxamine may be a therapy for patients with IVH or intraventricular extension after intracerebral hemorrhage. (Stroke. 2011;42:465-470.)

Key Words: deferoxamine ■ hydrocephalus ■ intraventricular hemorrhage ■ iron

Intraventricular hemorrhage (IVH) occurs in up to 50% of patients with primary intracerebral hemorrhage (ICH) and in 45% of patients with aneurysmal subarachnoid hemorrhage.1-3 Recent studies have found IVH is a predictor of poor outcome after ICH.4,5 According to the International Surgical Trial in ICH, hydrocephalus develops in 55% patients with IVH.1 That trial also showed that in ICH patients, having IVH lowers the favorable outcome from 31.4% to 15.1%, and that the presence of hydrocephalus lowers the likelihood of favorable outcome further to 11.5%. The mechanisms of IVH-induced hydrocephalus are not well-understood.

Iron overload occurs in the brain after ICH and has a key role in ICH-induced brain damage. It is known that increased brain iron levels contribute to brain edema, oxidative injury, and brain atrophy after ICH.4-6 Our previous studies showed that free iron levels in cerebral spinal fluid (CSF) increase almost 14-fold after ICH on the third day and remain high for at least 28 days after ICH.7 In patients with subarachnoid hemorrhage, CSF iron levels are approximately 10-fold higher than in control patients.8 Deferoxamine (DFX), an iron chelator, can reduce hemorrhagic and ischemic brain injury. Our recent studies have shown that DFX reduces ICH-induced brain injury in aged rats and piglets.9-11

Long-term ventricular dilatation has been previously observed in a rat model of IVH in our laboratory.12 We hypothesized that iron may have a role in hydrocephalus and brain damage after IVH. The current study examined that hypothesis and determined the effect of DFX on IVH-induced brain injury.

Materials and Methods

Animal Preparation and Intraventricular Injection

Animal use protocols were approved by the University of Michigan Committee on the Use and Care of Animals. A total 114 male Sprague–Dawley rats (Charles River Laboratories, Portage, MI), weighing 250 to 350 grams, were used in this study. Animals were anesthetized with pentobarbital (50 mg/kg intraperitoneal) and the right femoral artery was catheterized to monitor arterial blood pressure, blood pH, PaO₂, PaCO₂, hematocrit, and glucose levels. Core body temperature was maintained at 37.5°C with a feedback-controlled heating pad. Rats were then positioned in a stereotoxic frame (Kopf Instruments). A cranial burr hole (1 mm) was drilled and a 26-gauge needle was inserted stereotactically into the right lateral ventricle (coordinates: 0.6 mm posterior, 4.5 mm ventral, and 1.6 mm lateral to the bregma). Autologous arterial blood or saline was infused at a rate of 14 μL/min using a microinfusion pump (World Precision Instruments). The needle was removed after
injection, the burr hole was filled with bone wax, and the skin incision was closed with sutures.

Experimental Groups

These experiments were performed in 2 parts. In the first part, rats received an injection of 200 μL autologous whole blood or saline into the right lateral ventricle. Some rats (IVH, n = 5 to 7 per time point; saline control, n = 3 to 7 per time point) were euthanized at days 1, 3, 7, and 28 for immunohistochemistry. For the rats euthanized at day 28, serial MRI were performed at days 1, 3, 7, 14 and 28. For nonheme iron measurement and Western blot analysis, rats (nonheme iron, n = 6 for each group; Western blots, n = 4 for each group) were euthanized at days 1, 3, and 7. In the second part, rats received an injection of autologous whole blood (200 μL) into the right lateral ventricle and had DFX (100 mg/kg intramuscular, n = 9) or vehicle (same volume of saline, n = 8) treatment at 2 and 6 hours after IVH, and then every 12 hours for 7 days. MRI scans were conducted at days 1, 3, 7, 14 and 28 after IVH, and the rats were then euthanized for brain histology.

MRI and Volume Measurement

Imaging was performed in a 7.0-T Varian MR scanner (183-mm horizontal bore; Varian). Rats were anesthetized with 2% isoflurane/air mixture throughout MRI examination. The imaging protocol for all rats included a T2 fast spin-echo sequence (repetition time/echo time = 4000/60 ms) and a T2* gradient-echo sequence (repetition time/echo time = 250/5 ms). The field of view was 35 × 35 mm, and the matrix was 256 × 128 mm; 25 coronal slices (0.5-mm-thick) were acquired to cover the entire axis of the lateral ventricles. All image analysis was performed using Image J. Lateral ventricular volumes were calculated from T2 images as described before. Bilateral ventricles were outlined and the areas were measured. Ventricular volume was obtained by combining the ventricle areas over all slices, showing the lateral ventricles, and multiplying by section thickness (0.5 mm). Volume measurements were performed by a blinded observer. Volume of intraventricular hypointensity, representing hemorrhage and iron accumulation, was calculated from T2* gradient-echo MRI using the same method as for intraventricular volume. Hippocampal volume was also calculated from T2 images using this method with some modification. Bilateral hippocampal areas were outlined and measured. Hippocampal areas in 10 MRI slices, from the first slice to last slice (~2.12 to ~6.62 mm from bregma), were combined and multiplied by section thickness (0.5 mm) to calculate hippocampal volume.

Immunohistochemistry

Rats were anesthetized with pentobarbital (100 mg/kg intraperitoneal) and perfused with 4% paraformaldehyde in 0.1 mol/L pH 7.4 phosphate-buffered saline. 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 optimal cutting temperature compound (Sakura Finetek USA) and 18-μm-thick slices were cut using a cryostat. Immunohistochemical studies were performed using the avidin–biotin complex technique as
previously described. The primary antibodies were polyclonal rabbit anti-rat heme oxygenase-1 (HO-1) IgG (1:200 dilution; StressGene), polyclonal rabbit anti-human ferritin IgG (1:400 dilution; DAKO), and mouse anti-rat neuronal nuclei IgG (1:400 dilution; Chemicon International).

Cell Counts
Immunopositive cells were assessed on images taken by a digital camera using 2 sections at \( \approx -3.3 \) mm from bregma. Three high-power images (×40 magnification) per section were taken in the hippocampus or in the periventricular area. HO-1 and ferritin-positive cells were counted on the hippocampus and periventricular area. Neuronal nuclei-positive cells were counted on the hippocampus. Cell counts were performed by a blinded observer. All measurements were repeated 3 times and the mean value was used.

Western Blot Analysis
Western blot analysis was performed as previously described. The brains were perfused with saline before decapitation. The hippocampus and periventricular brain tissue (~1-mm-thick brain tissue around the ventricle) were sampled. The primary antibodies were polyclonal rabbit anti-rat HO-1 IgG (1:2000 dilution; StressGene), rabbit anti-ferritin heavy chain polyclonal antibody (1:2000 dilution; Cell Signaling Technology), and goat anti-ferritin light chain polyclonal antibody (1:2000 dilution; Abnova). The relative densities of bands were analyzed with NIH ImageJ. HO-1 and ferritin levels were expressed as percent of saline control.

Non-Heme Brain Tissue Iron Determination
Rats were euthanized at 1, 3, and 7 days after IVH or saline injection. The brains were perfused with saline before decapitation. Left and right hemispheres were sampled and weighed. Nonheme brain tissue iron was determined as previously described. Nonheme iron levels in IVH rats were expressed as percent of saline control.

Statistical Analysis
Values are given as means±SD. Student t tests or Mann-Whitney U tests were used to analyze the data. Differences were considered significant at \( P<0.05 \).

Results
Intraventricular injection of autologous arterial blood resulted in marked enlargement of cerebral lateral ventricles. Lateral ventricular volumes in IVH rats were much larger than in rats receiving saline injection at 24 hours (61.7±5.7 vs 8.4±1.3 mm³).
in saline group; \( P < 0.01; \) Figure 1A) through 4 weeks (40.4\( \pm \)6.8 vs 10.1\( \pm \)8.0 mm\(^3\); \( P < 0.01; \) Figure 1A).

Four weeks after IVH, hippocampal volumes were smaller in IVH rats compared to saline-injected controls (87.0\( \pm \)2.6 mm\(^3\) vs 102.2\( \pm \)5.1 mm\(^3\) in the saline group; \( P < 0.01; \) Figure 1B). There was also a significant reduction of neuronal nuclei-positive cells in the CA1 area of hippocampus in IVH rats (109\( \pm \)26 vs 165\( \pm \)40 cells/mm in saline group; \( P < 0.01 \)) at this time point. Although IVH caused neuronal death in the hippocampus, no mortality was found in this rat IVH model.

T2*-weighted MRI was used to examine iron accumulation after IVH. T2* lesions were mainly located in the lateral ventricle of the injection side at 1 day after blood injection. Thereafter, the T2* lesion volume progressively declined, but lesions were still observable at day 28, when they were mainly at the edge of the lateral ventricles (Figure 2A). Brain nonheme iron levels were significantly increased at days 3 and 7 (Figure 2B).

Brain HO-1 and ferritin levels were examined by Western blots and immunohistochemistry. Brain HO-1 levels were increased after IVH (Figure 3A). HO-1-positive cells were detected in the brain after either IVH or saline control, although they were much more prevalent after IVH (Figure 3). In IVH rats, HO-1-positive cells were increased at all the time points in the hippocampus and the periventricular area. Expression peaked at day 3 and then decreased with time (Figure 3). Most HO-1-positive cells were glia-like cells. Ferritin-positive cells were also significantly increased in the hippocampus and the periventricular area after IVH, and high expression levels were maintained for 4 weeks (Figure 4). Western blot analysis showed levels of ferritin light chain and ferritin heavy chain were increased after IVH (Figure 5).

DFX treatment (100 mg/kg intramuscular at 2 and 6 hours after IVH and then every 12 hours for 7 days) reduced lateral ventricular volume significantly at 2 weeks (34.0\( \pm \)8.9 vs 44.0\( \pm \)10.2 mm\(^3\) in vehicle-treated group; \( P < 0.05; \) and 4 weeks (32.7\( \pm \)10.6 vs 43.8\( \pm \)9.7 mm\(^3\) in vehicle-treated group; \( P < 0.05; \) Figure 6) after IVH. There was a trend of decreasing of intraventricular T2* lesion volume in DFX-treated compared to vehicle-treated rats from the first week, but the difference only reached significance at 4 weeks after IVH (7.8\( \pm \)1.9 vs 10.1\( \pm \)2.4 mm\(^3\); \( P < 0.05; \) Figure 6). Immunohistochemistry showed that DFX reduced ferritin-positive cells in the periventricular area (103\( \pm \)40 vs 168\( \pm \)54 cells/mm\(^2\) in vehicle-treated group; \( P < 0.05 \)) and hippocampus (54\( \pm \)16 vs 73\( \pm \)10 cells/mm\(^2\) in vehicle-treated group; \( P < 0.05 \)). In addition, DFX reduced IVH-induced hippocampal tissue loss and CA1 neuronal loss at 4 weeks after IVH (hippocampal volume: 89.0\( \pm \)2.7 vs 85.2\( \pm \)4.1 mm\(^3\); \( P < 0.05 \); CA1 neurons: 148\( \pm \)23 vs 120\( \pm \)29 cells/mm in the vehicle-treated group, \( P < 0.05 \)).

**Discussion**

The major findings of the current study are: (1) intraventricular injection of autologous whole blood causes iron accumulation, hydrocephalus, and brain tissue loss in the hippocampus; (2) HO-1 and ferritin levels are increased significantly in the hippocampus and periventricular areas; and (3) DFX, an iron chelator, reduces IVH-induced hydrocephalus and hippocampal tissue loss.

Limited studies on hydrocephalus after IVH have been performed in adult animal models. Pang et al\(^ {16} \) established an IVH model in adult dogs by injecting 9 mL of solid
autologous blood clots into the lateral ventricles. Long-term ventricular dilatation was achieved in an adult rat model of IVH established previously in our laboratory by intraventricular injection of 200 μL fresh autologous blood. However, persistent posthemorrhagic ventricular dilation was not evident in an adult pig model of IVH. Results from different IVH animal models suggest that multiple factors, including animal species and volume and rate of blood injection, are essential for hydrocephalus development. Our current results indicate that the intraventricular blood injection rat model is a reliable model to study hydrocephalus after IVH. Currently, no good behavioral tests are available for this IVH rat model, and this is a limitation of this study. Development of behavioral tests for IVH should be a priority.

Our T2* studies found iron deposition from the first day of IVH to 4 weeks later and that iron deposition is associated with ventricular dilation. T2* MRI is clinically used as a sensitive method to detect acute hemorrhage and iron deposition in old hemorrhagic lesions. Clinically, hyperacute IVH can be clearly visualized in T2* MRI, although there is little information available on subacute and long-term IVH with T2* MRI. Our recent study showed that T2* MRI sequences reflect brain tissue iron deposition after rat ICH.

The mechanisms of hydrocephalus development after IVH still are not clear. Although the relationship between iron (the degradation product of hemoglobin) in CSF and hydrocephalus is seldom studied, some clues indicate that there are connections between iron and posthemorrhagic hydrocephalus in clinical studies. Hemoglobin released from red cells reaches its peak concentration by the second day after injection of blood into the CSF. Nonprotein-bound iron was found frequently at higher levels in CSF from preterm infants with posthemorrhagic ventricular dilatation. In subarachnoid hemorrhage patients, a high level of ferritin in CSF is an independent predictive factor for chronic hydrocephalus. In addition, iron and ferritin deposition are found in ependymal or subependymal locations after neonatal IVH. Increases in CSF iron levels are present after various kinds of intracranial hemorrhage. However, to date, the relationship between iron level increases in CSF and ventricular dilatation is unknown. To our knowledge, there is only one article reporting iron and hydrocephalus in a dog model of subarachnoid hemorrhage. In 1960, Iwanowski et al injected an iron–dextran complex into the subarachnoid spaces repeatedly in 2 dogs. The 2 dogs died at day 3 and day 12 after injections. Our recent study showed that T2* MRI sequences reflect brain tissue iron deposition after rat ICH. At present, the mechanisms associated with ventricular dilatation induced by iron are unknown. Traditional explanations for posthemorrhagic hydrocephalus mainly include: (1) arachnoid obstruction by blood components; (2) subarachnoid fibrosis; (3) proliferation and fibrosis of the arachnoid villi; and (4) ventricular wall injury.

Activities of normal ependymal cilia is thought to direct CSF current toward the ventricular outlets, and absent or functionally defective ependymal cell motile cilia may be a cause of hydrocephalus in a mouse model. Fibrosis either in subarachnoid space or in arachnoid villi also has been suggested to have a role in the development of posthemorrhagic hydrocephalus. Despite a lack of studies on the relationship between subarachnoid fibrosis and iron, the association between iron overload and liver tissue fibrosis is well-known. Iron might also contribute to the long-term hydrocephalus that is found even after clot resolution by promoting periventricular tissue destruction (as found for the hippocampus).

Deferoxamine, an iron chelator, has been shown to be neuroprotective in rat and pig models of ICH. There is a current phase I (dose finding and safety study) clinical trial of DFX in ICH patients. In the present study, we demonstrated that DFX also can alleviate ventricular dilatation as well as neuronal loss in hippocampus after IVH. Accordingly, DFX
treatment reduced intraventricular T2* lesion volume and brain ferritin immunoreactivity. The dose of DFX used in this study is adopted from our previous ICH studies. Although DFX may work directly by chelating iron, it should be noted that DFX also can inhibit hemin-induced erythrocyte lysis.29

Conclusion
In conclusion, the present study revealed persistent ventricular dilatation together with evidence of iron accumulation in an adult rat model of IVH. Our results demonstrated the role of iron in IVH-induced hydrocephalus and brain damage. DFX may be a potential therapeutic to treat IVH patients, especially those ICH patients with ventricular extension.

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Disclosures
None.

References
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