Effects of Deferoxamine on Intracerebral Hemorrhage-Induced Brain Injury in Aged Rats

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Background and Purpose—Deferoxamine (DFX) reduces brain edema, neuronal death, and neurological deficits after intracerebral hemorrhage (ICH) in young rats. In the present study, we investigated whether DFX is effective on brain injury after ICH in aged rats and examined dose dependency.

Methods—Male Fischer 344 rats (18 months old) had an intracaudate injection of 100 μL autologous whole blood and were treated with different doses of DFX (10, 50, and 100 mg/kg) or vehicle 2 and 6 hours post-ICH and then every 12 hours up to 7 days. Rats were euthanized at Day 3 for brain edema determination and Day 56 for brain atrophy measurement. Behavioral tests were performed during the experiments.

Results—All 3 doses of DFX attenuated perihematomal brain edema at 3 days (eg, at dose 50 mg/kg, 80.4 ± 0.5% versus 81.6 ± 0.9% in the vehicle-treated group, P < 0.01). Fifty and 100 mg/kg DFX also reduced ICH-induced ventricle enlargement, caudate atrophy, and ICH-induced neurological deficits in aged rats. However, although 10 mg/kg DFX reduced ventricle enlargement and forelimb-placing deficits, it did not reduce caudate atrophy and corner turn deficits.

Conclusions—These results indicate that DFX can reduce ICH-induced brain injury in aged as well as young rats and that a dose > 10 mg/kg is the optimal dose of DFX in this model. (Stroke. 2009;40:1858-1863.)

Key Words: brain atrophy ■ cerebral hemorrhage ■ deferoxamine ■ iron

Intracerebral hemorrhage (ICH) is a common and often fatal stroke subtype. Many patients with an intracerebral hematoma experience progressive deterioration in their neurological condition due to formation of secondary brain edema. Even if the patients survive this acute phase, prolonged neurological deficits and brain atrophy commonly occur.

Iron, a heme degradation product, has an important role in ICH-induced brain injury. After erythrocyte lysis, iron concentrations in the brain can reach very high levels. We have shown a 3-fold increase of brain nonheme iron after intracerebral hemorrhage in rats, and brain iron levels remain high for at least several weeks. Deferoxamine (DFX), an iron chelator, can rapidly penetrate the blood–brain barrier and accumulate in the brain tissue at a significant concentration after systemic administration. Our previous studies have found that DFX reduces ICH or hemoglobin-induced brain edema, neuronal death, neurological deficits, and brain atrophy in young rats.

ICH is mostly a disease of the elderly, but current experimental ICH models have primarily used young animals. Age is an important factor affecting brain injury in ischemic stroke in animals and humans. Recently, we found that ICH caused greater neurological deficits, more severe brain swelling, greater induction of heat shock proteins, and enhanced microglial activation in aged rats compared with young rats.

Age is an important predictor of mortality after ICH in humans. These results suggest that age is a significant factor in determining brain injury after ICH.

In relation to iron-induced brain injury after ICH, aged rats have higher levels of heme oxygenase-1 after ICH. That enzyme is involved in the production of iron from heme during hematoma resolution and inhibitors of heme oxygenase-1 have been shown to reduce ICH-induced brain injury. In addition, increased microglial activation in aged rats may also impact iron handling. Apart from being the major site of heme oxygenase-1 expression after ICH, microglia also show a marked increase in L- and H-ferritin expression, major endogenous iron chelators, after ICH. These findings suggested that aging might impact the protective effects of DFX in ICH.

The present study, therefore, investigated whether DFX is effective on brain injury, including brain edema, prolonged neurological deficits, and brain atrophy, after ICH in aged rats. It also examined the optimal dose for DFX to reduce ICH injury.

Materials and Methods

Animal Preparation and Intracerebral Infusion

Animal use protocols were approved by the University of Michigan Committee on the Use and Care of Animals. A total of 99
18-month-old male Fischer 344 rats (weight, 380 to 450 g; National Institutes of Health, Bethesda, Md) were used in this study. Rats were anesthetized with pentobarbital (45 mg/kg intraperitoneally). The right femoral artery was catheterized for continuous blood pressure monitoring and blood sampling. Blood was obtained from the catheter for analysis of blood pH, PaO₂, PaCO₂, hematocrit, and blood glucose. Core temperature was maintained at 37°C with use of a feedback-controlled heating pad. Rats were positioned in a stereotactic frame (Kopf Instruments), and a cranial burr hole (1 mm) was drilled on the right coronal suture 3.5 mm lateral to the midline. A 26-gauge needle was inserted stereotactically into the right basal ganglia (coordinates: 0.2 mm anterior, 5.5 mm ventral, 3.5 mm lateral to the bregma). Autologous whole blood (100 µL) was injected at a rate of 10 µL/min using a microinfusion pump (Harvard Apparatus Inc). Sham controls had only intracerebral needle insertion. After injection, the needle was removed, the burr hole was filled with bone wax, and the skin incision was closed with sutures.

**Experimental Groups**

There were 2 sets of experiments in this study. In the first set, ICH rats were treated with DFX (10, 50, or 100 mg/kg administered intramuscularly; n = 9 for each dose) or vehicle (n = 14) 2 and 6 hours after ICH and then every 12 hours for 3 days. Sham rats were treated with 100 mg/kg DFX (n = 3) or vehicle (n = 6) after surgery as ICH rats. Rats were euthanized at Day 3 for brain edema measurement. In the second set, rats were treated with DFX (10, 50, or 100 mg/kg; n = 9 for each dose) or vehicle (n = 12) 2 and 6 hours post-ICH and then every 12 hours for 7 days. Sham rats were treated with 100 mg/kg DFX (n = 5) or vehicle (n = 5). Rats had T₂-weighted MRIs at 8 weeks after ICH, and then they were euthanized for histological analyses. Behavioral tests were undertaken on the day before surgery and then 1, 28, and 56 days after surgery. DFX administration was not blinded, but brain atrophy and neurological deficits were measured by an investigator who was blinded to the treatment status of the animal. Body weight and blood pressure were measured at 1, 3, 7, 14, 21, 28, 42, and 56 days after surgery. Body weight was expressed as percent change in body weight using the following formula: % change in body weight = (body weight on each time point − body weight before surgery)/body weight before surgery. Mean arterial blood pressure was measured using tail cuff plethysmography (ITC Life Science). The heat chamber was set at 29°C for optimal tail arterial dilatation to allow the measurement of the pulsatile pressure. A tail cuff/sensor was inflated by the system to a maximum pressure of approximately 150 mm Hg, and mean arterial blood pressure was determined using the optical sensor.

**Brain Water and Ion Contents**

Animals were anesthetized again and decapitated to measure brain water and ion contents. The rat brains were removed, and a coronal tissue slice (3-mm thick) 4 mm from the frontal pole was cut using a blade. The brain tissue slice was divided into 2 hemispheres along the midline, and each hemisphere was dissected into cortex and basal ganglia. The cerebellum served as a control. Five tissue samples from each brain were obtained: the ipsi- and contralateral cortex, the ipsi- and contralateral basal ganglia, and the cerebellum. Brain samples were dried at 100°C to obtain the dry weight. Samples were outlined and outlined areas were measured using ImageJ (Version 1.37v; National Institutes of Health). Brain samples were immediately weighted on an electric analytic balance (model AE 100; Mettler Instrument) to obtain the wet weight. Brain samples were dried at 100°C for 24 hours to obtain the dry weight. The formula for our calculations was the following: (wet weight − dry weight)/wet weight. Then the dehydrated samples were digested in 1 mL of 1 mol/L nitric acid for 1 week. Sodium and potassium contents of this solution were measured using the automatic flame photometer (model IL 943; Instrumentation Laboratory). Ion content was expressed in microequivents per gram of dehydrated brain tissue (mEq/kg dry wt).15

**Behavioral Tests**

ICH-induced neurological deficits were assessed using forelimb-placing and corner turn tests.4 In the vibrissae-elicted forelimb-placing test, animals were held by their bodies to allow the forelimbs to hang free. Independent testing of each forelimb was conducted by brushing the respective vibrissae on the corner of a tabletop once per trial for 10 trials. A score of 1 was given each time the rat placed its forelimb onto the edge of the table in response to vibrissae stimulation. The percentage of successful placing responses was determined for the impaired and the unimpaired forelimbs. For the corner turn test, the rat was allowed to proceed into a corner whose angle was 30°. To exit the corner, the animal could turn to either the left or the right, and the direction was recorded. This task was repeated 10 to 15 times and the percentage of right turns calculated.

**Magnetic Resonance Imaging**

MR scans were performed at 2 months after ICH. All rats were anesthetized with 1.5% to 2% isoflurane/air mixture throughout MRI examination. Rats lay prone head first in a 7.0-T Varian MR scanner (183-mm horizontal bore; Varian) with the body temperature maintained at 37°C using circulated heated air. A double-tuned volume radiofrequency coil was used to scan the head region of the rats. Axial T₂-weighted images were acquired using a fast spin-echo sequence with the following parameters: TR/effective TE, 4000/60 ms; field of view, 50×50 mm; matrix, 256×128; slice thickness, 1.0 mm; slice spacing, 0 mm; number of slices, 25; and number of scans, 1 (total scan time approximately 2 minutes.). Five MRI slices, from the slice showing the front tip of lateral ventricle to 5 mm posterior, were scanned on a computer. Then bilateral ventricles were outlined and outlined areas were measured using ImageJ (Version 1.37v; National Institutes of Health). Ventricles volume was obtained by combining the 5 ventricle areas and multiplying by the thickness (1 mm) of the sections. All measurements were repeated 3 times and the mean value was used. Ventricles volume was expressed as a percentage of the ipsilateral/contralateral.

**Brain Atrophy Measurement**

Rats were again anesthetized (intraperitoneal 60 mg/kg pentobarbital kg) and underwent transcardiac perfusion with 4% paraformaldehyde in 0.1 mol/L phosphate-buffered saline (pH 7.4). The brains were removed and kept in 4% paraformaldehyde for 6 hours and then immersed in 30% sucrose for 3 to 4 days at 4°C. Brains were then placed in optimal cutting temperature embedding compound (Sakura Finetek, Inc) and sectioned on a cryostat (18-µm thick slices). We estimated brain atrophy morphometrically. Coronal sections from 1 mm posterior to the blood injection site were stained with hematoxylin and eosin, and they were scanned. The bilateral caudate were outlined on a computer and caudate size measured as described in the MRI method. To minimize the influence of tissue shrinkage, brain atrophy was expressed as a percentage of the ipsilateral/contralateral area.

**Statistical Analysis**

All data in this study are presented as means±SD. Data were analyzed with Student t test or one-way analysis of variance. Differences were considered significant at P<0.05.

**Results**

**Physiological Variables**

All physiological variables were measured immediately before an intracerebral infusion. Mean arterial blood pressure, blood pH, PaO₂, PaCO₂, hematocrit, and blood glucose level were controlled within normal ranges (data not shown).

**Brain Edema**

The effect of DFX treatment on brain edema formation in aged rats was assessed 3 days after induction of ICH. ICH caused a marked increase in perihematomal water content in vehicle-treated rats (81.6±0.9 versus 77.7±0.4% in sham control, P<0.01). Systemic administration of 100 mg/kg DFX...
starting 2 hours after ICH reduced brain edema (80.3% ± 0.6% versus 81.6% ± 0.9% in the vehicle-treated group; P < 0.01). DFX at doses 50 and 10 mg/kg also reduced perihematomal brain edema (80.4% ± 0.5%, P < 0.01, and 80.7% ± 0.9%, P < 0.05, respectively; Figure 1A). The amelioration of ICH-induced edema formation with DFX was associated with a reduced accumulation of sodium and a reduced loss of potassium in the ipsilateral basal ganglia (Figure 1B-C). DFX treatment did not change brain water and ion contents in sham-operated rats (Figure 1).

Body Weight, Mean Arterial Blood Pressure, and Mortality

During the long-term experiments, body weight and mean arterial blood pressure were monitored. Rat body weight was decreased after surgery and the peak of body weight reduction was at Day 7. From that point, rat body weight gradually increased. DFX treatment did not affect the body weight change among the ICH groups. Also, in sham rats, there were no differences between DFX- and vehicle-treated groups (Figure 2).

As measured by tail cuff plethysmography, mean arterial blood pressure did not differ significantly during the time course of the experiments for any group (group mean values vary from 109 to 114 mm Hg) nor was it different between the experimental groups. Thus, there were no differences in blood pressure with DFX treatment in either the ICH or sham-operated rats nor were there differences between ICH and sham-operated rats.

Mortality rate in ICH rats was low in this study. There was one death at Day 33 in the vehicle-treated group, one death at Day 4 in the 10 mg/kg DFX group, and one death at Day 49 in the 50 mg/kg DFX group.

Neurological Deficits

Behavioral tests, including the forelimb-placing and the corner turn tests, were performed before ICH and 1, 28, and 56 days after ICH. In vehicle-treated ICH rats, a partial recovery of forelimb-placing occurred with time, but residual neurological deficits were still present at 56 days (64% ± 35% response rate). When rats were treated with DFX, ICH-induced forelimb-placing deficits showed a greater recovery (90% response rate at 28 and 56 days; Figure 3A). In the corner turn test, the percentage of turns to the right was significantly decreased at 28 days in ICH+DFX 10 mg/kg (68% ± 11%, P < 0.05), 50 mg/kg (61% ± 8%, P < 0.01), and 100 mg/kg (60% ± 7%, P < 0.01) treatment groups compared with the ICH+vehicle group (79% ± 10%). ICH-induced corner turn deficits were almost normalized (equal turns to right and left) at 56 days in DFX 50 mg/kg (56% ± 9% right turns) and 100 mg/kg (58% ± 8% right turns) treatment groups (Figure 3B). DFX 100 mg/kg treatment did not cause any neurological deficits in sham rats (Figure 3A–B).

Brain Atrophy

Rats had T2-weighted MRI at 2 months after ICH (Figure 4A). MRI showed the ipsilateral ventricle enlargement induced by ICH (600% ± 339% in ICH+vehicle group). DFX reduced ICH-induced ventricle volume enlargement (Figure 4B).
Ipsilateral caudate atrophy as compared with the contralateral side was found in coronal brain sections stained with hematoxylin and eosin at 2 months after ICH (Figure 5A). In vehicle-treated rats, the ipsilateral caudate was 75.8% of contralateral. DFX treatment with 50 mg/kg or 100 mg/kg significantly reduced caudate atrophy (83.6% and 84.3% of contralateral; \( P < 0.05 \) versus vehicle-treated ICH group). There was a tendency for a reduction in caudate atrophy in the 10 mg/kg group, but this did not reach significance (Figure 5B).

**Discussion**

In the present study, we found that DFX treatment attenuated ICH-induced brain edema, neurological deficits, and brain atrophy in aged rats without affecting body weight and mean arterial blood pressure. These results suggest that iron chelation may be a useful therapy for patients with ICH.

Aged Fischer rats (18 months old), which were commercially available from the National Institute on Aging, were used. The average lifespan of people is 72 years and the average lifespan of a male rat is between 2 and 3 years. As a percent of average lifespan, 18 months old in a rat corresponds to 50 years old in a human.

Based on the clinical experience with tissue plasminogen activator for acute ischemic stroke, we believed that 2 hours postictus is the earliest time point that DFX could be administered for ICH. Our results indicate that iron contributes to acute as well as delayed brain injury. In the acute phase, iron can potentiate thrombin toxicity in the brain. Given those results, starting DFX treatment at 2 hours would likely maximize induced protection.

The 3 major end points in our study were brain edema, brain atrophy, and neurological scores. Perihematomal edema is thought by many, but not all, to be a major cause of death and disability after ICH, particularly in relation to herniation. In combination with the presence of the hematoma, further mass effect due to edema formation can result in a midline shift and herniation. Perihematomal edema, as seen on CT scan, can result from clot retraction. However, that represents a redistribution of fluid between hematoma and
brain. A progressive mass effect can only result from movement of fluid from the blood to the brain either in the form of hematoma enlargement and/or progressive perihematoma edema. Although all agree that the latter does occur, its extent/prevalence has been debated. It is easy to examine where perihematoma tissue can be sampled and water content determined directly in animal models. In animals, there is substantial evidence for progressive edema formation.

Our studies on ICH have indicated that thrombin and iron are 2 major factors that are responsible for brain edema formation. Perihematoma brain edema is maximal at Day 3 in rats. We, therefore, tested the effects of deferoxamine on ICH-induced edema at Day 3. The degree of protection found with 100 mg/kg DFX, a reduction in water content from 81.6% to 80.3%, was very similar to that found in young rats (81.2% to 79.9%).

Brain atrophy has been found in animals and humans after ICH. The underlying cause(s) of this atrophy is, however, unknown. Our recent study suggested iron overload is associated with brain atrophy after ICH and deferoxamine reduces ICH-induced brain atrophy in young rats. Here we demonstrate that iron contributes to brain atrophy development after ICH in aged rats.

We found that DFX reduced ICH-induced neurological deficits in aged rats at 4 and 8 weeks. All 2 sensorimotor behavioral tests appear to be well suited to models of unilateral brain injury because they measure asymmetry. Thus, they can factor out confounding variables for behavioral tests such as decreased overall activity after surgery. These sensorimotor tests are also not altered by repeated testing and they do not require special training or food deprivation. The degree of improvement in neurological testing and they do not require special training or food content determined directly in animal models. In animals, there is substantial evidence for progressive edema formation.

DFX (10, 50, or 100 mg/kg) was given intramuscularly as suggested by Novartis Pharmaceuticals Corp. The maximal dose, 100 mg/kg, was chosen based on our previous studies, which indicated this dose was effective in reducing ICH-induced brain injury. The lower doses were chosen based on normal dosing in humans for other conditions. Deferoxamine is normally given as a 1000-mg dose followed by 500 mg every 4 to 12 hours if needed. The maximal recommended daily dose is 6000 mg. Assuming a body weight of 80 kg, the initial human dose would be 12.5 mg/kg, hence our lower dose of 10 mg/kg. An intermediate dose of 50 mg/kg was also chosen because of the possibility that ICH may require a higher therapeutic dose than a systemic disease.

In this study, we found that systemic treatment of 50 or 100 mg/kg DFX significantly reduced ICH-induced perihematoma brain edema at 3 days after ICH and reduced ipsilateral ventricular enlargement and caudate atrophy 2 months after ICH compared with the vehicle-treated rats with ICH. It also improved behavioral outcomes after ICH. On the other hand, the ICH rats treated with 10 mg/kg DFX showed significant brain edema reduction and attenuation of ipsilateral ventricle enlargement compared with vehicle-treated ICH rats; however, caudate atrophy was not significantly reduced, and residual neurological deficit was present at 56 days after ICH in the corner turn test. These results indicate that a dose >10 mg/kg is the optimal dose of DFX in this model.

DFX, an iron chelator, is a drug for the treatment of acute iron intoxication and of chronic iron overload due to transfusion-dependent anemias. DFX can rapidly penetrate the blood–brain barrier and accumulate in the brain tissue at a significant concentration after systemic administration. DFX chelates iron by forming a stable complex that prevents the iron from entering into further chemical reactions. However, DFX may cause hypersensitivity reactions; systemic allergic reactions; and cardiovascular, hematologic, and neurologic adverse reactions. Serious adverse reactions include significant hypotension and marked body weight loss. In the present study, all 3 of the DFX doses used did not cause hypotension and/or significantly affect body weight in the aged ICH rats.

In summary, systemic administration of DFX reduced ICH-induced brain edema, neurological deficits, and brain atrophy without causing severe side effects in aged rats. These results suggest that the protection offered by DFX against ICH-induced brain injury occurs irrespective of age and that iron chelation with DFX could be a new therapy for ICH.

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Disclosures
None.

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