Deferoxamine-induced attenuation of brain edema and neurological deficits in a rat model of intracerebral hemorrhage

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Object. Previous studies undertaken by the authors have indicated that iron accumulation and oxidative stress in the brain contribute to secondary brain damage after intracerebral hemorrhage (ICH). In the present study the authors investigate whether deferoxamine, an iron chelator, can reduce ICH-induced brain injury.

Methods. Male Sprague–Dawley rats each received an infusion of 100 µl of autologous whole blood into the right basal ganglia and were killed 1, 3, or 7 days later. Iron distribution was examined histochemically (enhanced Perls reaction). The effects of deferoxamine on ICH-induced brain injury were examined by measuring brain edema and neurological deficits. Immunohistochemical analysis was performed to investigate 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage, and Western blot analysis was performed to measure the amount of apurinic/apyrimidinic endonuclease/redox effector factor–1 (APE/Ref-1), a repair mechanism for DNA oxidative damage.

Iron accumulation was observed in the perihematomal zone from 1 day after ICH. Deferoxamine attenuated brain edema, neurological deficits, and ICH-induced changes in 8-OHdG and APE/Ref-1.

Conclusions. Deferoxamine and other iron chelators may be potential therapeutic agents for ICH. They may act by reducing the oxidative stress caused by the release of iron from the hematoma.

KEY WORDS • cerebral hemorrhage • iron • oxidation • brain edema • neurobehavioral outcome • deferoxamine

Abbreviations used in this paper: APE = apurinic/apyrimidinic endonuclease; ICH = intracerebral hemorrhage; MABP = mean arterial blood pressure; OCT = optimal cutting temperature; 8-OHdG = 8-hydroxy-2'-deoxyguanosine; RBC = red blood cell; Ref-1 = redox effector factor–1; SD = standard deviation.
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and water. The animals were anesthetized with pentobarbital (40 mg/kg given intraperitoneally), and the right femoral artery was catheterized to monitor arterial blood pressure and to sample blood for an intracerebral infusion. Blood pH, PaO₂, PaCO₂, MABP, hematocrit, and glucose values were monitored. Rectal temperature was maintained at 37.5°C by using a feedback-controlled heating pad. The rats were positioned in a stereotactic frame and a cranial burr hole (1 mm) was drilled near 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, and 3.5 mm lateral to the bregma). Autologous whole blood (100 μl) was infused at a rate of 10 μl/minute with the aid of a microinfusion pump. The needle was removed and the skin incision was closed with a suture after infusion.

Experimental Groups

This study was performed in three parts. In the first part, the two groups, the rats received an intracaudate injection of 100 μl autologous whole blood or merely a needle insertion (three rats for each time point). The rats were killed at 1, 3, and 7 days later. In Part 1 we evaluated the time course of iron accumulation after ICH. An enhanced Perls Prussian blue staining reaction was used for iron staining. In Part 2 we investigated APE/Ref-1 protein levels by performing a Western blot analysis (three rats for each time point). In Part 3 we investigated the effect of deferoxamine on APE/Ref-1, brain edema, and the animals’ behavior after ICH. Fifty-four rats received an intracaudate injection of 100 μl autologous whole blood and were treated with either deferoxamine (100 mg/kg in 1 ml saline administered intraperitoneally every 12 hours) or vehicle (1 ml saline administered intraperitoneally each time). The animals were divided into the following six groups according to the time of treatment onset after ICH: 1) deferoxamine or saline administered 2 hours after ICH and then at 12-hour intervals; 2) deferoxamine or saline administered 6 hours after ICH and then at 12-hour intervals; and 3) deferoxamine or saline administered 24 hours after ICH and then at 12-hour intervals until the day before the animals were killed. Three days after onset of ICH some animals were anesthetized and then killed for examination of brain edema (six rats in each group), immunohistochemical analysis (three rats in each group), and Western blot analysis (three rats in each group). Other animals underwent behavioral testing 1, 3, and 7 days after onset of ICH (six rats in each group).

Iron Staining

We used the Perls staining method to detect ferric iron. After the rats had been anesthetized, they underwent intracardiac 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 12 hours, after which they were immersed in 25% sucrose with 4% paraformaldehyde for 24 hours. The tissue was frozen in embedding OCT compound and sectioned on a cryostat (18 μm thick).

Sections of brain tissue were washed with distilled water and incubated in 1% H₂O₂ for 10 minutes. The sections were then incubated in 0.5% diaminobenzidine with nickel solution for 60 minutes.

Immunohistochemical Staining

Using the avidin–biotin complex technique, sections were incubated in 1:10 dilution of goat or horse serum for 30 minutes, rinsed, and incubated overnight with the primary antibody. The primary antibody was mouse anti–8-OHdG monoclonal antibody (10 μg/ml). Normal mouse immunoglobulin G was used as a negative control.

Western Blot Analysis

The rats were anesthetized before undergoing intracardiac perfusion with saline. The brains were then removed and a 3-mm-thick coronal brain slice was cut approximately 4 mm from the frontal pole. The slice was separated into ipsilateral and contralateral basal ganglia. Western blot analysis was performed as previously described. Briefly, 50 μg of proteins for each sample was separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a Hybond-C pure nitrocellulose membrane. The membrane was blocked in Carnation nonfat evaporated milk. The membrane was then probed with a 1:1000 dilution of the primary antibody (polyclonal rabbit anti–APE/Ref-1 antibody) and a 1:1500 dilution of the secondary antibody (peroxidase-conjugated goat anti–rabbit antibody). The antigen–antibody complexes were visualized with the aid of a chemiluminescence system and exposed to film. The relative densities of the bands were analyzed using Image software.

Content of Brain Water and Ions

For this experiment the rats were anesthetized (pentobarbital 50 mg/kg, intraperitoneal injection) and decapitated 3 days after ICH to determine the water and ion contents of the brain. The brains were removed and a blade was used to cut a coronal brain slice (~3 mm thick) that was located 4 mm from the frontal pole. The brain slice was divided into two hemispheres along the midline and each hemisphere was dissected into the cortex and the basal ganglia. The cerebellum also served as a control. Five samples from each brain were obtained: ipsilateral and contralateral cortex, ipsilateral and contralateral basal ganglia, and cerebellum. Brain samples were weighed immediately on an electronic analytical balance to obtain the wet weight. The brain samples were then 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. The dehydrated samples were digested in 1 ml of 1 mol/L nitric acid for 1 week, after which the Na⁺ and K⁺ contents of this solution were measured using a flame photometer. The ion content was expressed in milliequivalents per kilogram of dehydrated brain tissue.

Behavioral Tests

Corner turn and forelimb placing tests were used in this study. For the corner turn test, the rat was allowed to proceed into a corner, the angle of which was 30°. To exit the corner, the animal could turn either to the left or right, and the selection was recorded. This was repeated 10 to 15 times and the percentage of right turns was calculated.

Forelimb placing was scored using the vibrissae-elicited forelimb placing test. Each animal was held by its body to allow the forelimbs to hang free. Independent testing of each forelimb was induced by brushing the respective vibrissae against the corner of a table top once per trial for 10 trials. A score of one was given each time the rat placed its forelimb onto the edge of the table in response to the vibrissae stimulation. The percentage of successful placing responses was determined for both the impaired forelimb and the nonimpaired forelimb.

Statistical Analysis

All data in this study are presented as means ± SD. Data from the Western blot study and the tests of water and ion contents were analyzed using the Student t-test or a one-way analysis of variance, followed by the Scheffé post hoc test. A two-way analysis of variance was used to analyze the behavioral data and the significance of differences among groups was evaluated using the Scheffé post hoc test. A probability level lower than 0.05 was considered significant.

Sources of Supplies and Equipment

The animals, which had been obtained from Charles River Laboratories (Portage, MI), were positioned in a stereotactic frame purchased from Kopf Instruments (Tujunga, CA). The microinfusion pump used in the experiments was manufactured by Harvard Apparatus, Inc. (South Natick, MA). For the histological study, OCT compounds were purchased from Sakura Finetek, Inc. (Torrence, CA). For the immunohistochemical study the first antibody was monoclonal mouse anti–8-OHdG antibody (Oxis International, Inc., Portland, OR) and for the Western blot analysis the first antibody...
was rabbit polyclonal anti–APE/Ref-1 antibody (Novus Biologicals, Littleton, CO). Hybond-C pure nitrocellulose membranes and the chemiluminescence system used for the Western blot analysis were purchased from Amersham (Piscataway, NJ); Kodak X-OMAT film (Eastman–Kodak, Rochester, NY) was used. The relative densities of the bands in the immunoblot were analyzed with the aid of Image software (version 1.61), which is freely available from the US National Institutes of Health. The electronic balance (model AE 100) that was used to weigh tissue samples was obtained from Mettler Instrument Co. (Highstown, NJ) and the flame photometer (model IL 943) from Instrumentation Laboratory, Inc. (Lexington, MA).

Results

All physiological variables were measured immediately before intracerebral infusions. The MABP, pH, PaO₂, PaCO₂, hematocrit, and blood glucose level were controlled within normal ranges (MABP, 8–120 mm Hg; PaO₂, 80–120 mm Hg; PaCO₂, 35–45 mm Hg; hematocrit, 38–43%; blood glucose level, 80–120 mg/dl). The corner turn test and the forelimb placing test were scored by investigators (Y. H. and T. S.) who were blinded to treatment conditions.

Release of iron from the breakdown of hemoglobin occurred during intracerebral hematoma formation in the rat. By using the enhanced Perls reaction, iron-positive cells were found in the perihematomal zone as early as the 1st day post-ICH (Fig. 1A). Perls-positive cells were neuron-like on the 1st day and glialike several days later (Fig. 1B and C). A few Perls-positive cells were found in the cortex. There were no Perls-positive cells in the contralateral basal ganglia (Fig. 1D–F) and no positive cells in the ipsilateral basal ganglia in the sham-injury groups (data not shown). The effect of deferoxamine treatment was assessed 3 days after induction of ICH. Systemic administration of
Deferoxamine, when begun 2 hours after onset of ICH, reduced the water content of the brain in the ipsilateral cortex and basal ganglia (79.4 ± 0.3% compared with 80.5 ± 0.8%, p < 0.05, and 80 ± 0.9% compared with 81.8 ± 1.1%, p < 0.05, respectively; Fig. 2A). Deferoxamine treatment, when delayed for 6 hours after onset of ICH, also attenuated brain edema in the ipsilateral cortex (79.1 ± 0.7% compared with 80.1 ± 0.6% in vehicle-treated animals, p < 0.01; Fig. 2B). When begun 24 hours after onset of ICH, however, deferoxamine treatment failed to reduce brain edema (Fig. 2C). The amelioration of ICH-induced edema formation with deferoxamine was associated with a reduced accumulation of Na⁺ and a loss of K⁺ in the ipsilateral basal ganglia (Fig. 3A, B, D, and E). Deferoxamine had no effect on ion content in the brain when treatment was begun 24 hours after onset of ICH (Fig. 3F).

Deferoxamine treatment initiated 2 hours after ICH also ameliorated neurological deficits. The forelimb placing score was improved from 3 days post-ICH compared with the vehicle group (Day 3: 52 ± 17% compared with 12 ± 13%, p < 0.01; and Day 7: 60 ± 17% compared with 22 ± 15%, p < 0.01; respectively, Fig. 4A). A gradual improvement in ICH-induced corner turn asymmetry was also associated with deferoxamine therapy, with a significant improvement observed 7 days after ICH compared with the vehicle group (72 ± 19% compared with 95 ± 12%, p < 0.05; Fig. 4B).

FIG. 4. Bar graphs demonstrating the effect of deferoxamine treatment on behavior deficits following ICH. Forelimb placing test (A) and corner turn test (B) scores were measured before ICH and at 24 hours, 3 days, and 7 days after ICH (an infusion of 100 µl autologous whole blood), or in sham-injured controls (needle insertion without infusion). Animals that sustained ICH received either deferoxamine or saline starting at 2 hours after ICH and then every 12 hours. Values are expressed as means ± SDs; there were six animals in each group. *p < 0.01 compared with the sham-injured group; #p < 0.01 compared with the vehicle-treated group.

FIG. 5. Photomicrographs showing 8-OHdG immunoreactivity in the ipsilateral (A) and contralateral (C) basal ganglia of vehicle-treated rats and in the ipsilateral (B) and contralateral (D) basal ganglia of deferoxamine-treated rats 3 days after ICH. The deferoxamine treatment was initiated 2 hours after ICH. Bar = 20 µm.

FIG. 6. A: Western blot demonstrating APE/Ref-1 content in the contralateral (lanes 1–3) and ipsilateral (lanes 4–6) basal ganglia 3 days after ICH. Equal amounts of protein (50 µg) were used for each sample. B: Bar graph showing the results of the Western blot analysis of the time course of APE/Ref-1 expression in the contralateral and ipsilateral basal ganglia. Values are expressed as means ± SDs; there were three animals in each group. *p < 0.05 and **p < 0.01 compared with the contralateral basal ganglia.
Discussion

The results of the present study confirm that iron accumulates in the brain after ICH and that systemic deferoxamine treatment reduces ICH-induced brain edema and neurological deficits. Deferoxamine also reduces 8-OHdG immunoreactivities and ameliorates a decline in APE/Ref-1 levels in the brain after ICH; from this we may infer that deferoxamine reduces iron-mediated oxidative DNA damage. These results indicate that iron may contribute to oxidative brain damage after ICH and that iron is a target for ICH treatment.

Although iron is essential for normal brain function, an overload of the element can cause brain injury. After ICH, iron concentrations in the brain can reach very high levels due to the lysis of RBCs. Usually, most RBCs start to lye several days after ICH; however, RBC lysis can occur very early. For example, hemoglobin levels reach their peak by the 2nd day after injection of blood into the cerebrospinal fluid. In the present study, iron-positive cells were found in the perihematomal zone as early as the 1st day by an enhanced Perl reaction.

The current study showed that delayed (6 hours or less) iron chelation with deferoxamine attenuated perihematomal edema and neurological deficits, indicating that deferoxamine could be a therapeutic agent for ICH. Historically, brain water content has been expressed as a percentage. This can lead to a misunderstanding of the importance of changes in water content. For example, in the current data set, 3 days after ICH the water content of the contralateral basal ganglia was approximately 77.8%, whereas the water content of the ipsilateral basal ganglia was 81.8% in controls and 80% in deferoxamine-treated animals. A water content of 77.8% means that there is 3.5 g of water per gram of dry tissue. A water content of 81.8% means that there is 4.5 g of water per gram of dry weight. Thus, assuming that the amount of dry tissue does not change, this represents a 29% increase in water or a 22% increase in tissue mass (change in water + dry weight) in the basal ganglia on the ipsilateral side compared with that on the contralateral side. In the deferoxamine-treated group, an 80% water content means that there is 4 g of water per gram of dry weight, and this represents only a 14% increase in water compared with that found on the contralateral side, or an 11% increase in tissue mass. Thus, tissue swelling is reduced by half by deferoxamine treatment.

In animal models of stroke, the inclusion of a behavioral investigation and the data derived from it represents an important step forward, because a potential therapeutic compound should have a positive effect on behavior and function after stroke. We have used several sensorimotor behavioral tests to examine ICH-induced neurological deficits. Here, deferoxamine also improved both forelimb placing and corner turn scores.

In vitro studies have shown that in the brain deferoxamine reduces hemoglobin-induced Na/K-adenosine triphosphate inhibition and neuronal toxicity. Deferoxamine can penetrate the blood–brain barrier and accumulate in brain tissue at a significant concentration quickly after subcutaneous injection. The initial half-life of deferoxamine after intravenous infusion is 0.28 hours and the terminal half-life is 3.05 hours. In vivo, deferoxamine can reduce hemoglobin-induced brain edema.

Immunoreactivity of 8-OHdG was detected in the perihematomal area 3 days after ICH in animals treated with vehicle (Fig. 5A). In animals that received deferoxamine treatment 2 hours after ICH, however, little immunoreactivity of 8-OHdG was found in the ipsilateral basal ganglia (Fig. 5B). There was no immunoreactivity detected in the contralateral basal ganglia with (Fig. 5D) or without (Fig. 5C) deferoxamine treatment.

Protein levels of APE/Ref-1 in the ipsilateral basal ganglia were measured using Western blot analysis (Fig. 6A and B). These levels began to decrease as early as 24 hours after ICH (91 ± 3% of the contralateral basal ganglia, p < 0.01). The APE/Ref-1 protein levels were strongly reduced on Day 3 (15 ± 8%, p < 0.01) and this persisted on Day 7 (76 ± 15%, p < 0.05). With deferoxamine treatment, however, the APE/Ref-1 protein levels in the ipsilateral basal ganglia were significantly higher than those in the ipsilateral basal ganglia of animals treated with vehicle (4868 ± 148 pixels compared with 1101 ± 441 pixels, p < 0.01; Fig. 7) 3 days after ICH. Cortical levels of APE/Ref-1 (Western blot analysis) were normal after ICH and deferoxamine had no effect on these cortical levels.
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Previous studies on cerebral ischemia, brain injury, or hemoglobin toxicity\textsuperscript{1,4,8,15} have tended to include deferoxamine as a single dose (50–500 mg/kg administered either intraperitoneally or intravenously) before or immediately after the insult. We chose to inject deferoxamine at a dose of 100 mg/kg intraperitoneally every 12 hours because we previously found that a single dose of 50 mg/kg did not reduce brain injury after an intracerebral infusion of hemoglobin.\textsuperscript{8} We also chose repetitive drug administration because of the likelihood that iron would be released gradually from the hematoma as the RBCs lysed.

Iron can stimulate the formation of free radicals, leading to neuronal damage. Ferric and ferrous iron react with lipid hydroperoxides to produce free radicals.\textsuperscript{29} It is well known that iron reacts with lipid hydroperoxides to produce free radicals. Deoxyribonucleic acid is vulnerable to oxidative stress\textsuperscript{8,10} and 8-OHdG has been used as a marker of oxidative DNA damage.\textsuperscript{8,10} The immunoreactivity of 8-OHdG was observed after ICH in the present study. This result indicates that oxidative DNA damage is involved in hemorrhagic brain injury. The DNA repair enzyme APE/Ref-1 is responsible for repairing AP sites in DNA.\textsuperscript{14} Our results show that the level of APE/Ref-1, which is constitutively expressed in the noninjured brain, is significantly reduced after ICH. The decreased APE/Ref-1 protein levels after an insult to the brain indicate an oxidative DNA injury after ICH. Such a decrease in APE/Ref-1 levels has been found in other forms of brain injury associated with oxidative stress.\textsuperscript{2,14} The fact that the reduction in APE/Ref-1 is ameliorated in deferoxamine-treated animals indicates that the drug is reducing DNA oxidative damage, probably by reducing free radical production. It has been reported that antioxidative treatments improve neurological deficits in a rat ICH model induced by intracerebral injection of collagenase.\textsuperscript{22,23} It is still controversial, however, whether oxidative brain injury is a therapeutically target in human ICH. For example, in a human ICH study the levels of protein carbonyl and antioxidants in the perihematomal zone were not different from those in control brain tissue.\textsuperscript{17}

Although deferoxamine is an iron chelator, it can have other effects. Thus, it can act as a direct free radical scavenger\textsuperscript{6,15} and can induce brain tolerance.\textsuperscript{25} The latter has been demonstrated in vivo and in vitro and it may be related to a deferoxamine induction of hypoxia-inducible binding of transcription factor–1 to DNA.\textsuperscript{25}

The results of our previous studies have indicated that thrombin, hemoglobin, and hemoglobin degradation products are major factors responsible for ICH-induced brain edema formation. Thrombin is responsible for acute perihematomal brain edema and we have postulated that hemoglobin and its degradation products contribute to delayed brain edema.\textsuperscript{8} Nevertheless, the finding of iron-positive cells around the clot on the 1st day post-ICH indicates that iron may be released from RBCs during clot formation and play a role in acute edema formation. Indeed, it is interesting that, although deferoxamine was effective in reducing brain injury when given soon after ICH, it was ineffective when dosing began at 24 hours. Clot resolution in the rat\textsuperscript{19,38} and human\textsuperscript{20} takes days to weeks, indicating that there should be a gradual release of iron over that period. One potential reason why deferoxamine-inhibitable injury does not appear over a longer period is that the naturally occurring iron chelator, ferritin, is upregulated after ICH,\textsuperscript{31} presumably to limit iron-mediated damage.

Conclusions

After ICH, iron released from RBCs plays a major role in early brain injury. Deferoxamine has potential as a therapeutic agent for ICH, perhaps in combination with a thrombin antagonist such as argatroban, which also reduces early perihematomal edema in the rat.\textsuperscript{12}

References


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