

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

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Object. In the authors' previous studies they found that brain iron accumulation and oxidative stress contribute to secondary brain damage after intracerebral hemorrhage (ICH). In the present study they investigated whether deferoxamine, an iron chelator, can reduce ICH-induced brain injury.

Methods. Male Sprague–Dawley rats received an infusion of 100 μ l of autologous whole blood into the right basal ganglia and were killed 1, 3, or 7 days thereafter. Iron distribution was examined histochemically (enhanced Perl reaction). The effects of deferoxamine on ICH-induced brain injury were examined by measuring brain edema and neurological deficits. Apurinic/aprimidinic endonuclease/redox effector factor-1 (APE/Ref-1), a repair mechanism for DNA oxidative damage, was quantitated by Western blot analysis.

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

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

KEY WORDS • intracerebral hemorrhage • iron • oxidation • brain edema • deferoxamine

Spontaneous ICH is a frequent fatal subtype of stroke. Many patients harboring an intracerebral hematoma deteriorate progressively because of secondary brain edema formation. In our previous studies we demonstrated that toxic factors, including thrombin and hemoglobin, released from a blood clot may account for perihematoma edema formation.^{7,12,33}

Iron, a hemoglobin degradation product, is associated with lipid peroxidation and free radical formation in the brain after ICH.²⁸ Oxidative DNA damage has been found in the brain after ICH.³⁰ Iron overload plays an important role in many kinds of brain injury such as Alzheimer disease²⁶ and Parkinson disease.¹⁵ Considering the potential for massive iron overload in ICH, it is surprising that it has not been extensively studied as a therapeutic target.

Deferoxamine, an iron chelator, is used to treat hemochromatosis caused by iron toxicity.¹ Favorable effects

of iron chelator therapy have been reported in various models of cerebral ischemia.^{8,14} In our previous study we found that deferoxamine reduces hemoglobin-induced brain edema.⁷

Given the hypothesis that iron released from the clot contributes to brain injury following ICH, we have chosen to examine the effect of systemic deferoxamine treatment on brain edema and neurological deficits. We also studied the effects of deferoxamine on ICH-induced changes in APE/Ref-1, a multifunctional protein in the DNA base excision repair pathway responsible for repairing apurinic/aprimidinic sites in DNA after oxidative DNA damage.^{2,13} Reductions in this protein have been found in forms of brain injury associated with oxidative stress,¹³ and we hypothesized that such changes in ICH might be prevented by treatment with deferoxamine.

MATERIALS AND METHODS

Animal Preparation and Infusion

Animal protocols were approved by the University of Michigan Committee on the Use and Care of Animals. A

Abbreviations used in this paper: APE/Ref-1 = apurinic/aprimidinic endonuclease/redox effector factor-1; ICH = intracerebral hemorrhage; RBC = red blood cell; SD = standard deviation.

total of 81 male Sprague–Dawley rats, each weighing 300 to 400 g, were used for all experiments. Rats were allowed free access to food and water. The animals were anesthetized by intraperitoneal injection of pentobarbital (40 mg/kg), and the right femoral artery was catheterized to monitor arterial blood pressure and to sample blood for intracerebral infusion. Blood pH, PaO₂, PaCO₂, hematocrit, and glucose levels were monitored. Rectal temperature was maintained at 37.5°C by using a feedback-controlled heating pad. The rats were positioned in a stereotaxic frame, and a 1-mm cranial burr hole 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 by using a microinfusion pump. The needle was removed, and the skin incision was closed using suture after infusion.

Experimental Groups

This study was performed in three parts. All rats received a 100-µl intracaudate injection of autologous whole blood or a needle insertion. In part 1 we reevaluated the time course of iron accumulation after ICH. The three rats were killed at 1, 3, and 7 days thereafter, respectively. Enhanced Perl reaction was used for iron staining. In part 2 we investigated the effect of deferoxamine on brain edema and behavior after ICH. Rats received a 100-µl intracaudate injection of autologous whole blood and were treated with either deferoxamine (100 mg/kg in 1 ml saline intraperitoneally for 12 hours) or vehicle (1 ml saline intraperitoneally each time). The animals were divided into the following six groups according to the time of treatment onset after ICH: 1) deferoxamine or 2) saline administered 2 hours after ICH and then in 12-hour intervals; 3) deferoxamine or 4) saline administered 6 hours after ICH and then in 12-hour intervals; and 5) deferoxamine or 6) saline administered 24 hours after ICH and then in 12-hour intervals until the day before being killed. Some animals were anesthetized and then killed 3 days after ICH for brain edema examination (six in each group). Other rats underwent behavioral testing 1, 3, and 7 days after ICH (six in each group). In part 3 we investigated APE/Ref-1 protein levels by using Western blot analysis (three rats at each time point). The three rats were killed 1, 3, and 7 days later. In addition, the effect of 2-hour delayed deferoxamine treatment (100 mg/kg intraperitoneally every 12 hours for 3 days) on APE/Ref-1 levels was also tested. Three control rats received saline injection and were killed on Day 3 for Western blot analysis.

Iron Staining

In this study, Perl staining for ferric iron was performed.²⁹ Rats were anesthetized and underwent intracardiac perfusion with 4% paraformaldehyde in 0.1 mol/L (pH 7.4) phosphate-buffered saline. The brains were removed and kept in 4% paraformaldehyde for 12 hours and then immersed in 25% sucrose for 3 to 4 days at 4°C. The brains were then placed in OCT (optimum cutting temperature) embedding compound and 18-µ-thick sections were obtained on a cryostat.

After the sections were washed with distilled water and incubated in Perl solution (1:1, 5% potassium ferrocyanide and 5% hydrochloric acid) for 45 minutes, they were washed in distilled water six times for 5 minutes each.

In using diaminobenzidine with nickel enhancement of Perl staining we detected iron-positive cells without the use of free-floating sections. The Perl-stained sections were incubated in 0.5% diaminobenzidine with nickel solution for 60 minutes.

Western Blot Analysis

Animals were anesthetized before undergoing intracardiac perfusion with saline. The brains were removed and a 3-mm-thick coronal brain slice was cut approximately 4 mm from the frontal pole. The slice was separated into ipsi- and contralateral basal ganglia. Western blot analysis was performed as previously described.³² Briefly, 50-µg proteins for each were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a Hybond-C pure nitrocellulose membrane. The membranes were blocked in Carnation nonfat milk. Membranes were probed with a 1:1000 dilution of the primary antibody (polyclonal rabbit anti–APE/Ref-1 antibody) and a 1:1500 dilution of the second antibody (peroxidase-conjugated goat anti–rabbit antibody). The antigen–antibody complexes were visualized using a chemiluminescence system and exposed to film. The relative densities of bands were analyzed using an NIH Image system.

Brain Water and Ion Contents

Animals received an anesthetic of pentobarbital (50 mg/kg intraperitoneally) and were decapitated 3 days after ICH to determine brain water and ion contents.³¹ The brains were removed, and a coronal 3-mm-thick brain slice 4 mm from the frontal pole was cut with a blade. 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: the ipsi- and contralateral cortex, the ipsi- and the contralateral basal ganglia, and the cerebellum. Brain samples were immediately weighed to obtain the wet weight. Brain samples were then dried at 100°C for 24 hours to obtain the dry weight. The formula for calculation was as follows: (wet weight – dry weight)/wet weight. The dehydrated samples were digested in 1 ml of 1 mol/L nitric acid for 1 week. The sodium and potassium contents of this solution were measured using a flame photometer. Ion content was expressed in milliequivalents per kilogram of dehydrated brain tissue.

Behavioral Tests

The corner turn and forelimb placing tests were used in this study.⁵ In 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 this was recorded. The test 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.^{5,24} Animals were held by their bodies to allow their forelimbs to hang free. Independent

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testing of each forelimb was induced by brushing the respective vibrissae on the corner of a table top 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 the vibrissae stimulation. The percentage of successful placement responses was determined for impaired forelimb and nonimpaired forelimb.

Statistical Analysis

All data in this study are presented as the mean \pm SD. Data obtained in the Western blot analysis and water and ion contents were analyzed using the Student t-test or one-way analysis of variance, followed by the Scheffé post hoc test. Two-way analysis of variance was used to analyze the behavioral data, and significance of differences among groups was evaluated using the Scheffé post hoc test. Significance levels were measured at a probability value less than 0.05.

Sources of Supplies and Equipment

The animals, 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 histological examination, OCT (optimum cutting temperature) compounds were purchased from Sakura Finetek, Inc. (Torrence, CA). In 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 were purchased from Amersham (Piscataway, NJ), and Kodak X-OMAT film (Rochester, NY) was used. The relative densities of bands in immunoblot were analyzed with NIH Image version 1.61; NIH, Bethesda, MD. The electronic balance (model AE 100) 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. Mean arterial blood pressure, pH, arterial PaO₂ and PaCO₂ tensions, hematocrit, and blood glucose were controlled within the normal range (mean arterial blood pressure, 80–120 mm Hg; PaO₂, 80–120 mm Hg; PaCO₂, 35–45 mm Hg; hematocrit, 38–43%; blood glucose 80–120 mg/dl).

Release of iron from the breakdown of hemoglobin occurred during intracerebral hematoma formation. In the enhanced Perl reaction, iron-positive cells were found in the perihematoma zone as early as the 1st day (Fig. 1A). Perl-positive cells were neurons on the 1st day and glial cells several days later (Fig. 1B and C). There were no Perl-positive cells in the contralateral basal ganglia (Fig. 1D–F) nor in the ipsilateral basal ganglia in sham-treated groups (data not shown).

Systemic administration of deferoxamine starting 2 hours after ICH reduced brain water content in the ipsilat-

eral cortex and basal ganglia 3 days after ICH ($79.4 \pm 0.3\%$ compared with $80.5 \pm 0.8\%$ [$p < 0.05$]; $80 \pm 0.9\%$ compared with $81.8 \pm 1.1\%$ [$p < 0.05$], respectively) (Fig. 2A). Deferoxamine treatment delayed for 6 hours after ICH also attenuated brain edema in the ipsilateral cortex and the ipsilateral basal ganglia 3 days after ICH ($79.1 \pm 0.7\%$ compared with $80.1 \pm 0.6\%$ in vehicle-treated animals [$p < 0.05$]; $79.7 \pm 0.3\%$ compared with $81.5 \pm 0.7\%$ in vehicle-treated animals [$p < 0.01$], respectively) (Fig. 2B). Deferoxamine treatment starting 24 hours after ICH, however, failed to reduce brain edema at 3 days (Fig. 2C). The deferoxamine-related amelioration of ICH-induced edema formation was associated with reduced sodium ion accumulation and potassium ion loss in the ipsilateral basal ganglia (Fig. 3A, B, D, and E). Deferoxamine had no effect on brain ion content when treatment was instituted 24 hours after ICH (Fig. 3C and F).

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

Ipsilateral basal ganglia APE/Ref-1 protein levels were measured using Western blot analysis (Fig. 5A and B). The APE/Ref-1 levels started to decrease as early as 24 hours after ICH ($91 \pm 3\%$ of the contralateral basal ganglia [$p < 0.01$]). It was strongly reduced by Day 3 ($15 \pm 8\%$ of contralateral side, $p < 0.01$), and reduction persisted at 7 days ($76 \pm 15\%$ of contralateral side [$p < 0.05$]). With 2-hour delayed deferoxamine treatment, however, APE/Ref-1 protein levels in the ipsilateral basal ganglia were significant higher than those of the ipsilateral basal ganglia in vehicle-treated rats (4868 ± 148 pixels compared with 1101 ± 441 pixels [$p < 0.01$]) (Fig. 6) 3 days after ICH.

DISCUSSION

The findings in 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 ameliorates a decline in APE/Ref-1 levels in the brain after ICH, suggesting that it reduced iron-mediated oxidative DNA damage. These results indicate that iron may contribute to oxidative brain damage after ICH and that iron is a target in ICH treatment.

Although iron is essential for normal brain function, iron overload can cause brain injury. After ICH, iron concentrations in the brain can reach very high levels following RBC lysis.^{6,28} Usually, most RBCs start to lyse several days after ICH. Red blood cell lysis, however, can occur very early. For example, hemoglobin levels reach their peak by the 2nd day after injection of blood into the cerebrospinal fluid.¹⁶ In the present study, iron-positive cells

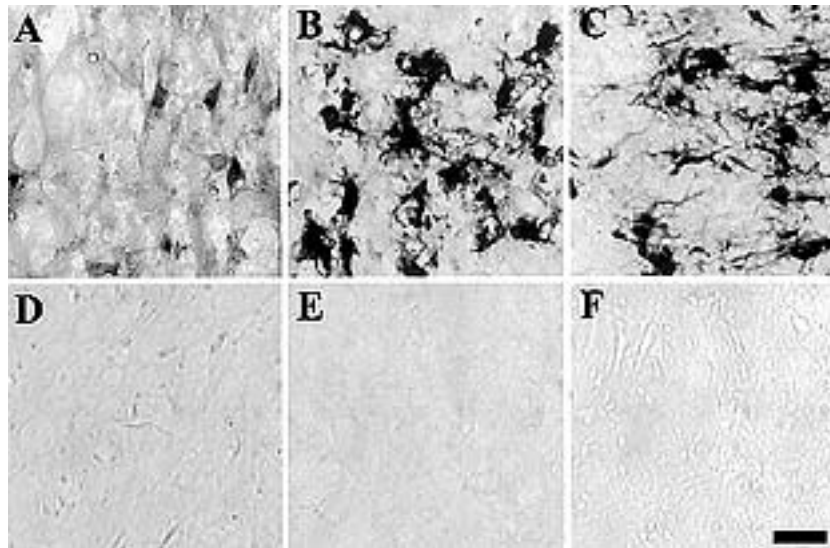


Fig. 1. Iron histochemistry by Perl staining in the ipsilateral (A–C) and contralateral (D–F) basal ganglia 1 (A and D), 3 (B and E), and 7 (C and F) days after ICH. Bar = 20 μ m. Magnification \times 400.

were found in the perihematoma zone as early as the 1st day, as detected by enhanced Perl reaction.

The current study showed that delayed (\leq 6 hours) iron chelation with deferoxamine attenuated perihematoma edema and neurological deficits, suggesting that deferoxamine could be a therapeutic agent for ICH. In animal models of stroke, the inclusion of data obtained in behavioral investigations is an important step forward, because a potential therapeutic compound should positively affect behavior and function after stroke. We have used several sensorimotor behavioral tests to examine ICH-induced neurological deficits.⁵ Here, deferoxamine also improved both forelimb placing and corner turn scores.

The authors of *in vitro* studies have shown that deferoxamine reduces hemoglobin-induced brain Na^+/K^+ adenosine triphosphate inhibition and neuronal toxicity.^{22,23} Deferoxamine can penetrate the blood–brain barrier and accumulate in the brain tissue at a significant concentration quickly after subcutaneous injection.^{10,19} The initial

half-life of deferoxamine after intravenous infusion is 0.28 hours, and the terminal half-life is 3.05 hours.²⁰ *In vivo*, deferoxamine can reduce hemoglobin-induced brain edema.⁷

In previous studies of cerebral ischemia, brain injury, or hemoglobin toxicity,^{3,7,14,27} investigators have tended to administer deferoxamine as a single 50- to 500-mg/kg intraperitoneal or -venous dose before or immediately after the insult. We chose to use an intraperitoneal 100-mg/kg dose of deferoxamine every 12 hours because we previously found that a single 50-mg/kg dose did not reduce brain injury following intracerebral infusion of hemoglobin.⁷ We also chose repetitive drug administration because of the likelihood that iron would be released gradually from the hematoma as RBCs lyse.

Iron can stimulate the formation of free radicals, leading to neuronal damage. Ferric and ferrous iron react with lipid hydroperoxides to produce free radicals.²⁵ It is well known that iron reacts with lipid hydroperoxides to pro-

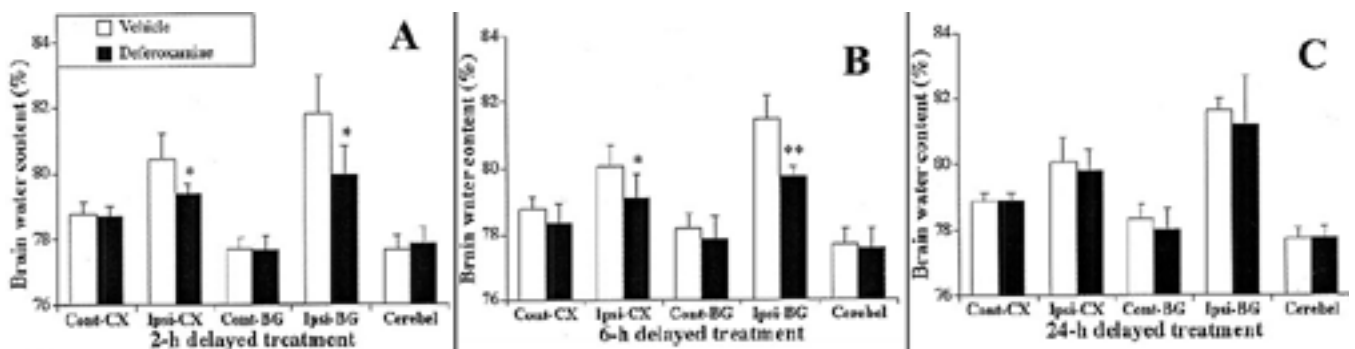


Fig. 2. Bar graphs demonstrating the effects of deferoxamine treatment on brain water content 3 days after ICH. There were six rats in each group. Measurements were made in brains obtained from rats treated with deferoxamine or vehicle 2 (A), 6 (B), or 24 (C) hours after ICH. Values are expressed as the means \pm SD. * p < 0.05 and ** p < 0.01 indicating differences from vehicle groups. Cerebel = cerebellum; Cont-BG = contralateral basal ganglia; Cont-CX = contralateral cortex; Ipsi-BG = ipsilateral basal ganglia; Ipsi-CX = ipsilateral cortex.

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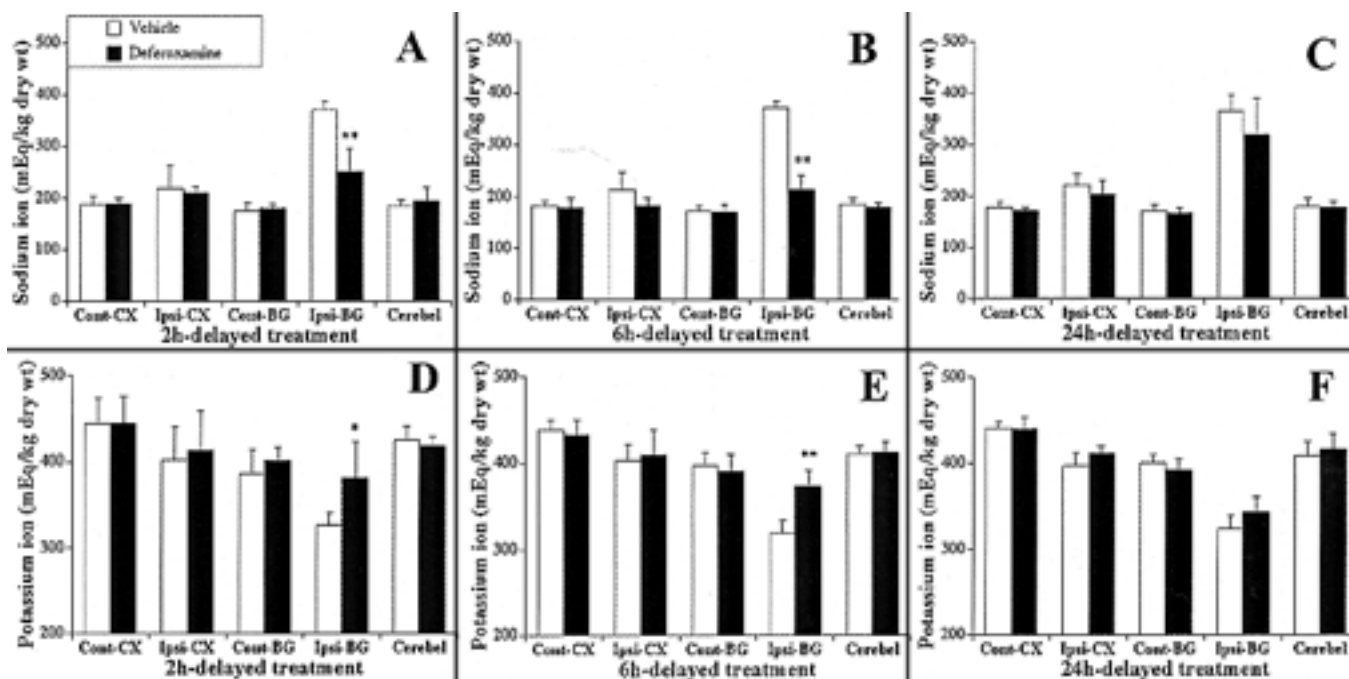


Fig. 3. Bar graphs showing the effects of deferoxamine treatment on sodium (A–C) and potassium (D–F) content 3 days after ICH. There were six rats in each group. Measurements were made in brains obtained from rats treated with deferoxamine or vehicle 2 (A and D), 6 (B and E), or 24 (C and F) hours after ICH. Values are expressed as the means \pm SD. * $p < 0.05$ and ** $p < 0.01$ indicating differences from vehicle groups.

duce free radicals. Furthermore DNA is vulnerable to oxidative stress.^{4,9} Apurinic/aprimidinic sites are hallmark of oxidative DNA damage. A DNA repair enzyme, APE/Ref-1, is responsible for repairing apurinic/aprimidinic-sites in DNA.¹³ Our results showed that APE/Ref-1, which is constitutively expressed in the noninjured brain, is significantly reduced after ICH. The decreased APE/Ref-1 protein levels after insult suggests post-ICH oxidative

DNA injury. Such a decrease in APE/Ref-1 has been found in other forms of brain injury associated with oxidative stress.^{2,13} The fact that the reduction in APE/Ref-1 is ameliorated in deferoxamine-treated animals suggests a reduction in DNA oxidative damage, probably by reducing free radical production.

Although deferoxamine is an iron chelator, it can have other effects. Thus, it can act as a direct free radical scav-

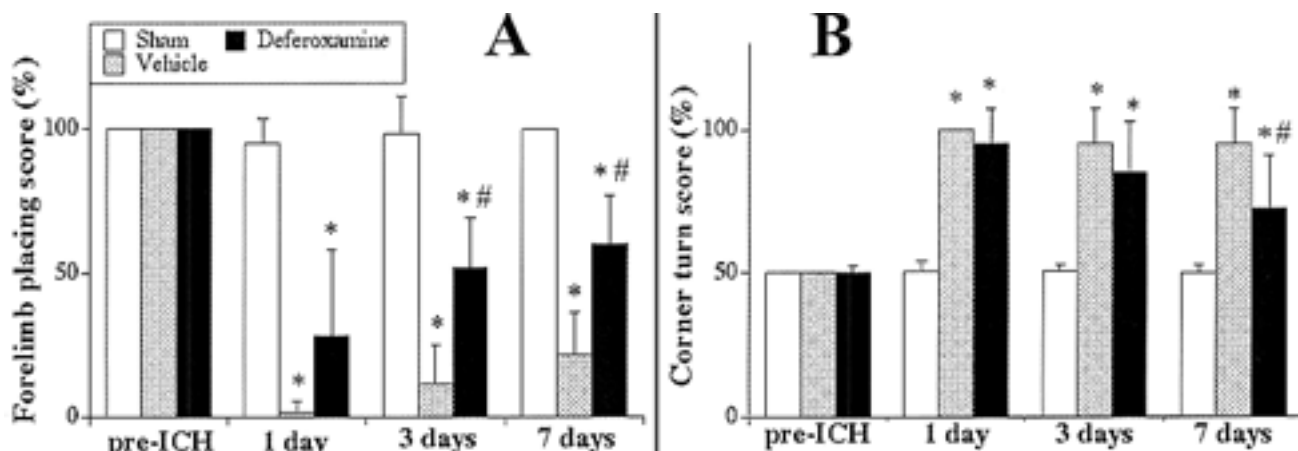


Fig. 4. Bar graphs illustrating the effects of deferoxamine treatment on behavior deficits following ICH. Forelimb placing (A) and corner turn test (B) scores were measured before ICH and at 24 hours, 3 days, and 7 days after ICH (an infusion of 100 μ l autologous whole blood) or in sham controls (needle insertion without infusion). The animals received either deferoxamine or saline starting at 2 hours after ICH and then every 12 hours. Values are expressed as the means \pm SD. There were six animals in each group. * $p < 0.01$ compared with the sham group; # $p < 0.01$ compared with the vehicle group.

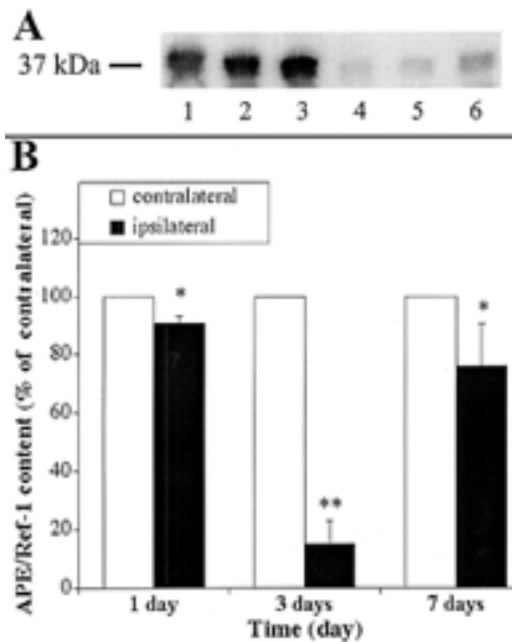


Fig. 5. A: Results of the Western blot analysis showing APE/Ref-1 content in the contralateral (Lanes 1–3) and ipsilateral (Lanes 4–6) basal ganglia 3 days after ICH. Equal amounts of protein (50 mg) were used. B: Graphic representation of the Western blot analysis results of the time course of APE/Ref-1 expression in the contra- and ipsilateral basal ganglia (three rats in each group). Values are the means \pm SD; * $p < 0.05$ and ** $p < 0.01$ indicating differences from the contralateral basal ganglia.

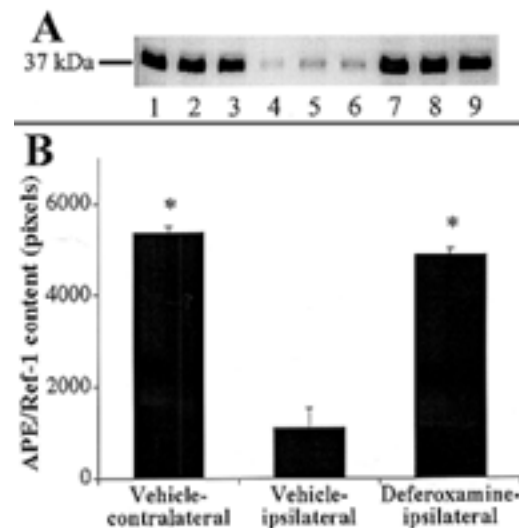


Fig. 6. The effects of 2-hour delayed deferoxamine treatment on APE/Ref-1 expression following ICH. A: Results of Western blot analysis showing APE/Ref-1 concentration in the vehicle-treated contralateral (Lanes 1–3), vehicle-treated ipsilateral (Lanes 4–6), and deferoxamine-treated ipsilateral (Lanes 7–9) basal ganglia 3 days after ICH. Equal amounts of protein (50 mg) were used. B: Graphic representation of Western blot analysis results of APE/Ref-1 expression in different rat group basal ganglia 3 days after ICH (three animals in each group). Values are expressed as the means \pm SD. * $p < 0.01$ compared with the vehicle-treated ipsilateral basal ganglia.

enger^{8,14} and it can induce brain tolerance.²¹ The latter has been demonstrated in vivo, and in vitro and it may be related to a deferoxamine induction of hypoxia-inducible transcription factor 1 binding to DNA.²¹

In our previous studies the findings have indicated that thrombin, hemoglobin, and hemoglobin degradation products are major factors responsible for ICH-induced brain edema formation. Thrombin is responsible for acute perihematoma brain edema, whereas we have postulated that hemoglobin and its degradation products contribute to delayed brain edema.⁷ Iron-positive cells found around the clot on the 1st day indicate that iron may release from RBCs during clot formation and function in acute edema formation. Indeed, it is interesting that although deferoxamine was effective in reducing brain injury when given soon after the ICH, it was ineffective when infused at 24 hours. That clot resolution in the rat^{17,34} and human¹⁸ takes days to weeks suggests 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,²⁸ 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 perihematoma edema in the rat.¹¹

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