

Long-term effects of experimental intracerebral hemorrhage: the role of iron

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Object. Intracerebral hemorrhage (ICH) causes brain atrophy and neurological deficits. The mechanisms of brain atrophy after ICH are poorly understood, although recent evidence suggests that some ICH-induced brain injury results from the products of hemoglobin degradation, including iron. In this study the authors examine the role of iron in brain atrophy and neurological deficits following ICH.

Methods. Male Sprague–Dawley rats received an infusion of either 100 μ l autologous whole blood or saline into the right caudate. Hematoxylin and eosin staining was used for histological examination, and iron levels and ferritin immunoreactivities were also examined. Deferoxamine was used as an iron chelator. Over the duration of the experiment, the rats underwent behavioral testing (forelimb placing, forelimb use asymmetry, and corner turn tests).

Brain atrophy in the caudate with prolonged neurological deficits occurred after ICH. Although partial functional recovery occurred with time, residual neurological deficits were still detectable at 3 months postprocedure. Iron accumulation and ferritin upregulation were present in the ipsilateral caudate. Deferoxamine reduced brain atrophy and improved behavioral outcomes, and it also reduced brain ferritin immunoreactivity.

Conclusions. An ICH results in an accumulation of iron in the brain that is not cleared within 3 months and that contributes to brain tissue loss and neurological deficits posthemorrhage. Iron chelation may be a useful therapy for patients with ICH.

KEY WORDS • intracerebral hemorrhage • brain atrophy • iron • ferritin •
deferoxamine • rat

INTRACEREBRAL hemorrhage often causes immediate death. If the patient survives the ictus, the hematoma gradually resolves within several months and restoration of function is usually incomplete.¹⁵ Prolonged neurological deficits and brain atrophy commonly occur in patients with ICH.²⁷ Animal models have been widely used to study the acute effects of the disease. The long-term effects of ICH in these models, however, are not well known. Recently, we demonstrated that several behavioral tests can be used to assess brain injury after ICH,¹¹ but it is not clear how long the ICH-induced behavioral deficits last in rats. Although delayed brain atrophy has been found in animal models of ICH, the mechanism causing brain atrophy after ICH is unknown.^{7,35}

Erythrocyte lysis and hemoglobin toxicity contribute to brain edema and cell death after ICH.^{13,30,31,34,36} Iron, one of the hemoglobin degradation products, plays a key role in neurodegenerative diseases.^{9,29} Iron overload can cause brain injury through many pathways, such as lipid peroxidation and the formation of free radicals.²⁶ We have demonstrated that iron overload occurs in the brain after ICH.³³

Abbreviations used in this paper: ICH = intracerebral hemorrhage; SD = standard deviation.

Ferritin, a naturally occurring iron chelator, is involved in maintaining iron homeostasis in the brain. Ferritin levels are upregulated in the brain after ICH,^{17,33} and they may limit iron-induced brain injury.

Our previous studies have demonstrated that deferoxamine attenuates acute ICH- and hemoglobin-induced brain edema.^{13,20} In this study we examined whether brain atrophy accompanied by long-term neurological deficits occurs after ICH in rats, and whether iron accumulation contributes to brain atrophy and neurological deficits. A preliminary report of these results has been presented in abstract form.¹²

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 42 male Sprague–Dawley rats weighing 300 to 350 g (Charles River Laboratories, Portage, MI) were used in this study. The animals were anesthetized with pentobarbital (45 mg/kg delivered intraperitoneally). The right femoral artery was catheterized for continuous blood pressure monitoring and blood sampling. Blood was obtained from the catheter for analysis of pH, PaO₂, PaCO₂, hematocrit, and glucose levels. The animals' core temperature was maintained at 37°C with

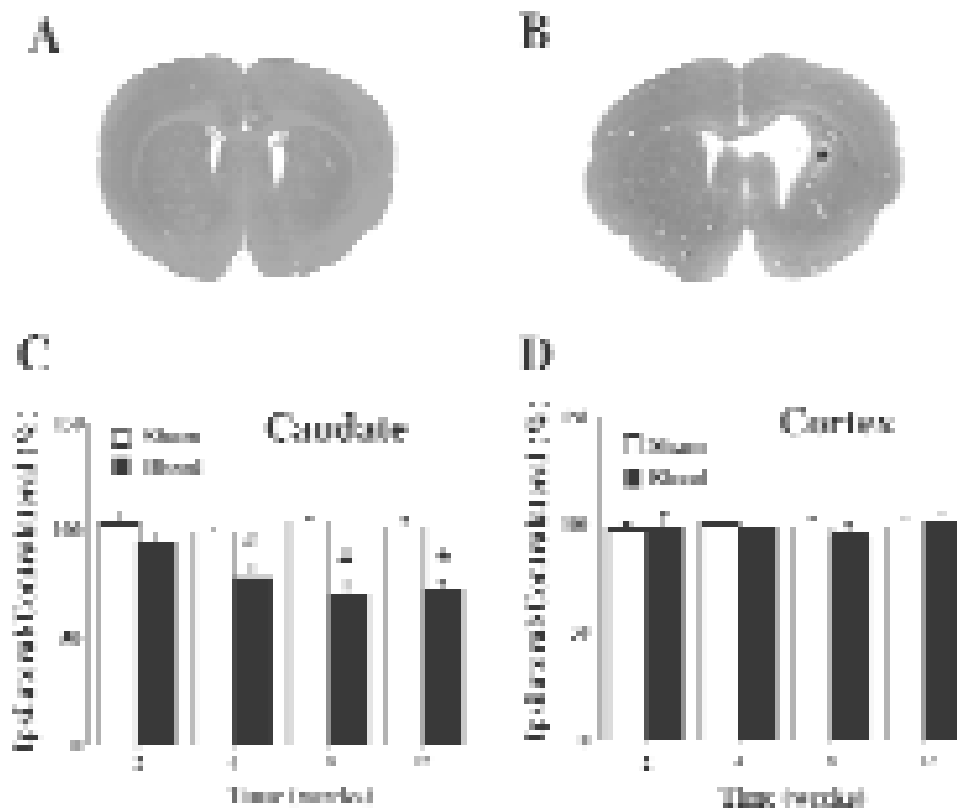


FIG. 1. A and B: Brain sections showing atrophy after ICH. Coronal gross sections obtained 4 weeks after 100- μ l injections of saline (sham operation, A) or autologous blood (B). Asterisk indicates the site of blood injection. C and D: Bar graphs showing brain volumes in rats with ICH and in sham-operated animals. Caudate volume was measured at 2, 4, 8, and 12 weeks after infusion of 100 μ l autologous blood or saline into the right caudate (C). Cortex volume was measured at 2, 4, 8, and 12 weeks after a 100- μ l infusion of either saline or autologous blood into the right caudate (D). Values for graphs are given as the means \pm SDs, with four or five rats in each group (* $p < 0.05$ and # $p < 0.01$, compared with saline control). H & E (A and B).

the use of a feedback-controlled heating pad. The rats were positioned in a stereotaxic frame (Kopf Instruments, Tujunga, CA), and a cranial bur hole (1 mm) was drilled on the right coronal suture 3.5 mm lateral to the midline. Autologous whole blood (100 μ l) was infused into the right caudate at a rate of 10 μ l/minute through a 26-gauge needle (coordinates: 0.2 mm anterior, 5.5 mm ventral, and 3.5 mm lateral to the bregma) with the aid of a microinfusion pump (Harvard Apparatus, Inc., South Natick, MA). In sham-operated animals 100 μ l saline was infused. The needle was removed, the bur hole was filled with bone wax, and the skin incision was sutured shut.

Experimental Groups

These experiments were performed in two parts. In the first, rats received a 100- μ l infusion of autologous whole blood or saline (18 rats in each group). The animals were then used for behavioral testing conducted at 2, 4, 8, and 12 weeks after ICH (four or five rats in each group). They were killed immediately after testing, and brain sections were prepared for H & E staining, immunohistochemical testing, and enhanced Perls reaction.

In the second part of the study, the rats received 100- μ l infusions of autologous whole blood and then underwent treatment with deferrioxamine (100 mg/kg administered intraperitoneally every 12 hours for 7 days, starting 2 hours post-ICH) or vehicle (same volume of saline given intraperitoneally; six rats in each group). Behavioral tests were performed at 2 and 4 weeks. The animals were then killed to obtain brain atrophy and ferritin immunoreactivity measurements.

Histological Studies: Immunohistochemistry and Histochemistry

The rats were anesthetized with pentobarbital (60 mg/kg administered intraperitoneally) and perfused with 4% paraformaldehyde in 0.1 M pH 7.4 phosphate-buffered saline. The brains were removed and kept in 4% paraformaldehyde for 4 to 6 hours, then immersed in 25% sucrose for 3 to 4 days at 4°C. The brains were embedded in optimal cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA) and 18- μ m-thick slices were cut using a cryostat.

We estimated brain atrophy morphometrically.⁷ Coronal sections from 1 mm posterior to the blood injection site were stained with H & E, then they were scanned. The caudate, cortex, and lateral ventricle were outlined on a computer, and the outlined areas were measured using the NIH Image program (version 1.62; National Institutes of Health, Bethesda, MD). All measurements were repeated three times and the mean value was used. To minimize the influence of tissue shrinkage, brain atrophy was expressed as a percentage of the contralateral area.

Immunohistochemical studies were performed using the avidin-biotin complex technique as previously described.³⁷ The primary antibody was polyclonal rabbit anti-human ferritin immunoglobulin G (DAKO, Glostrup, Denmark) at a 1:400 dilution. Normal rabbit immunoglobulin G was used for a negative control. Histochemical evaluation of iron levels was performed with Perls staining. Sections were washed with distilled water and incubated in a modified Perls solution (1:1; 5% potassium ferrocyanide and 5% hydrochloric acid).³³ Diaminobenzidine with nickel enhancement of the Perls reaction was able to detect iron-positive cells without the use of free-

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floating sections. The sections treated with Perls stain were incubated in 0.5% diaminobenzidine with nickel solution for 60 minutes.

Cell Counts

To examine the effects of deferoxamine on brain ferritin immunoreactivity after ICH, we used 18 μm -thick coronal sections from the blood injection site. Two high-power images (magnification $\times 200$) were obtained using a digital camera. One image was taken from the caudate just next to the cavity, and the other was taken from the primary motor cortex. Ferritin-positive cells were counted by a blinded observer. Counts were performed on two areas in each of six deferoxamine-treated or vehicle-treated rat brain sections.

Behavioral Tests

All animals were tested before and after surgery, and scores were assigned by investigators who were blinded to both neurological and treatment conditions. The following three behavioral tests were used: 1) the forelimb placing test; 2) the forelimb use asymmetry (cylinder) test; and 3) the corner turn test.¹¹

Forelimb Placing Test. Forelimb placing was scored using a vibrissae-elicited forelimb placing test.²⁴ Independent evaluation of each forelimb was induced by brushing the vibrissae ipsilateral to that forelimb on the edge of a tabletop once per trial for 10 trials (intact animals placed the forelimb quickly onto the countertop). The percentage of successful placing responses was determined, and we found that there is a reduction in successful responses in the forelimb contralateral to the site of injection after ICH.¹¹

Forelimb Use Asymmetry Test. Forelimb use during exploratory activity was analyzed by videotaping rats in a transparent cylinder for 3 to 10 minutes, depending on the degree of activity during the trial.²⁴ Behavior was quantified by determining the occasions when the unimpaired forelimb (ipsilateral, designated I) was used, calculated as a percentage of the total number of observations of limb use on the wall of the cylinder; the occasions when the impaired forelimb (contralateral to the blood injection site, designated C) was used, calculated as a percentage of the total number of observations of limb use on the wall of the cylinder; and the occasions when both forelimbs (designated B) were used simultaneously, calculated as a percentage of the total number of observations of limb use on the wall of the cylinder. A single overall limb use asymmetry score was calculated as follows: $I/(I + C + B) - C/(I + C + B)$.

Corner Turn Test. The rat was allowed to proceed into a corner, the angle of which was 30° . To exit the corner, the rat could turn either to the left or to the right, and this choice was recorded.²⁵ This test was repeated 10 to 15 times, with at least 30 seconds between each trial, and the percentage of right turns was calculated. Only turns involving full rearing along either wall were included. The rats were not picked up immediately after each turn so that they did not develop an aversion to turning around.

Statistical Analysis

The Mann-Whitney U-test was used to compare behavioral and brain atrophy data. Values are given as the means \pm SDs, with statistical significance set at a probability level of less than 0.05.

Results

Physiological data were measured prior to intracerebral blood infusion. The values of blood pH, blood gases, mean arterial blood pressure, hematocrit, and blood glucose levels were controlled in normal ranges (PO_2 , 80–120 mm Hg; PCO_2 , 35–45 mm Hg; pH, 7.4–7.5; mean arterial blood pressure, 70–110 mm Hg; hematocrit, 35–45%; blood glucose 6–9 mM).

The rats received a 100- μl infusion of autologous whole blood or saline, and they were killed 2, 4, 8, or 12 weeks later. Hematoxylin and eosin staining was used for histological examination of brain sections. At 2 weeks after ICH,

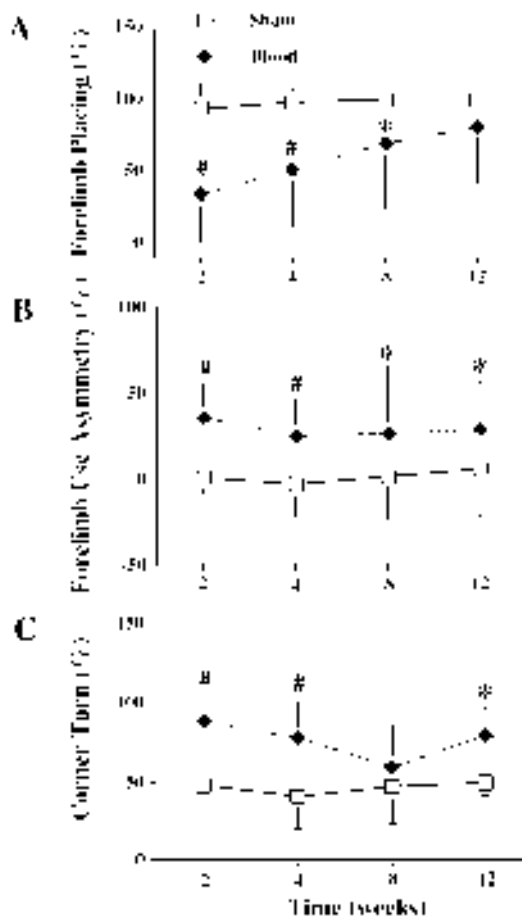


FIG. 2. Graphs showing results of behavioral tests administered after induction of ICH. The forelimb placing (A), forelimb use asymmetry (B), and corner turn tests (C) were performed at 2, 4, 8, and 12 weeks after sham operation or 100- μl autologous blood infusion into the right caudate. For a normal rat, the expected scores on these tests are 100, 0, and 50%, respectively. Values are given as the means \pm SDs, with five to 15 rats tested (* $p < 0.05$ and # $p < 0.01$, compared with saline-infused controls, Mann-Whitney U-test).

slight atrophy of the caudate appeared but did not reach significance ($p > 0.05$, Fig. 1). Nevertheless, there was significant caudate atrophy at 4 weeks ($78.3 \pm 6.9\%$ of the contralateral caudate compared with $98.1 \pm 2.8\%$ in the sham-operated control; four rats; $p < 0.05$), and there was enlargement of the ipsilateral lateral ventricle ($296 \pm 184\%$ of the contralateral lateral ventricle compared with $130 \pm 47\%$ in the sham-operated control; four rats; $p < 0.05$). Between 8 and 12 weeks, the ipsilateral caudate area was approximately 70% of the contralateral. In contrast to the caudate, cortical atrophy did not occur at any time.

Behavioral deficits were measured at 2, 4, 8, and 12 weeks after ICH. A partial recovery of forelimb placing occurred with time, but residual neurological deficits (forelimb placing, forelimb use asymmetry, and corner turn) were still detectable at 12 weeks (Fig. 2).

To examine iron clearance after ICH, we used the Perls reaction. At 4, 8, and 12 weeks after ICH, cells positive for Perls staining were detectable in the brain (Fig. 3). Ferritin immunoreactivity was very strong in the ipsilateral hemisphere at 4 weeks and was still elevated 8 to 12 weeks after

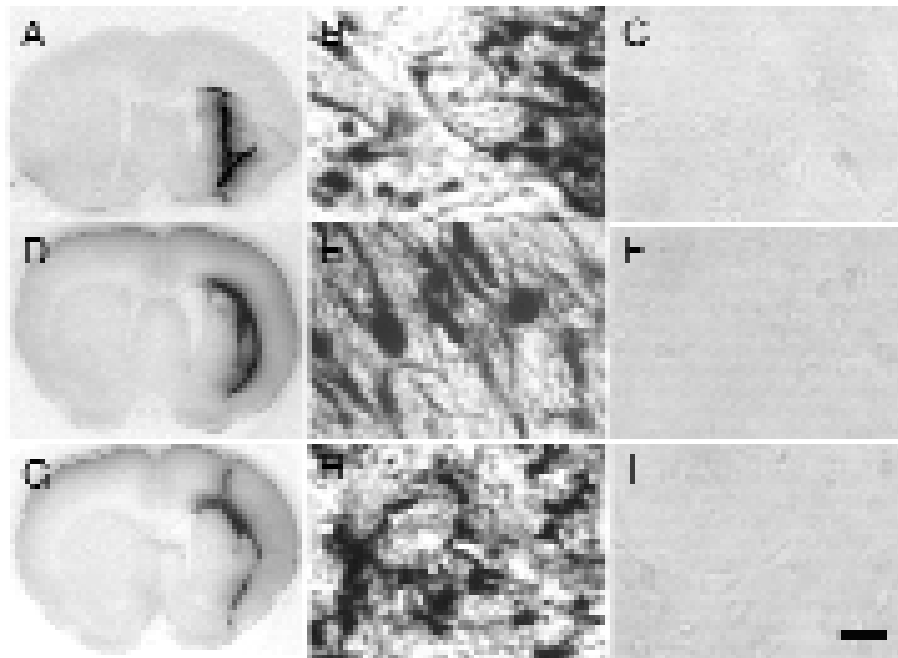


FIG. 3. Images of brain sections and photomicrographs showing Perls staining for ferric iron in the ipsilateral (B, E, and H) and contralateral (C, F, and I) caudate at 4 (A, B, and C), 8 (D, E, and F), and 12 (G, H, and I) weeks after induction of ICH by intracerebral injection of 100 μ l autologous blood. Panels A, D, and G are coronal gross sections. Bar = 20 μ m.

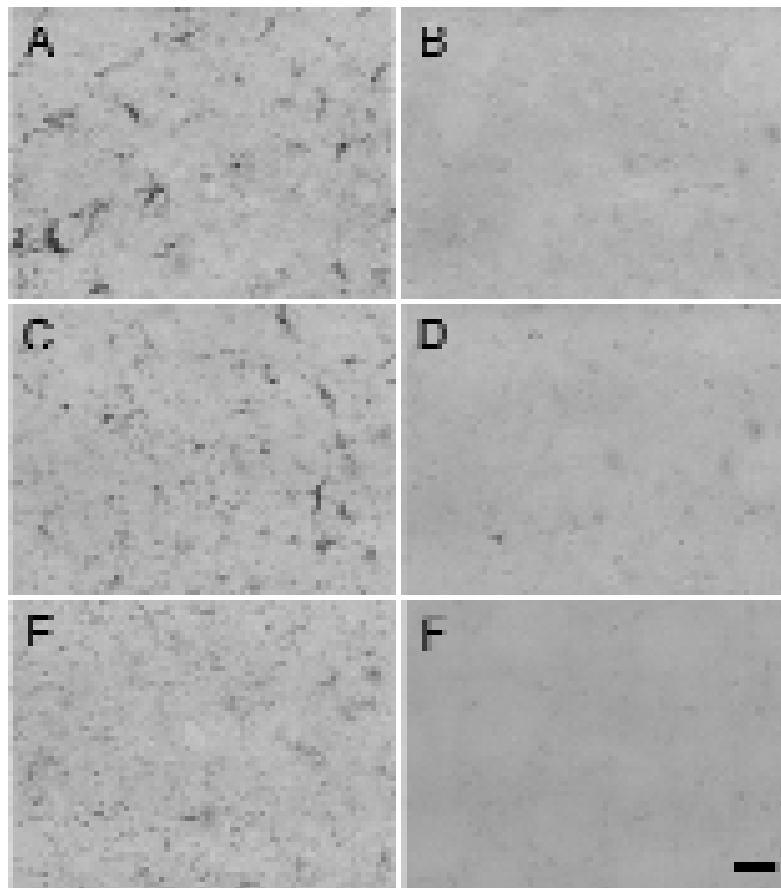


FIG. 4. Photomicrographs showing ferritin immunoreactivity in the ipsilateral (A, C, and E) and contralateral (B, D, and F) caudate 4 (A and B), 8 (C and D), and 12 (E and F) weeks after ICH. Bar = 20 μ m.

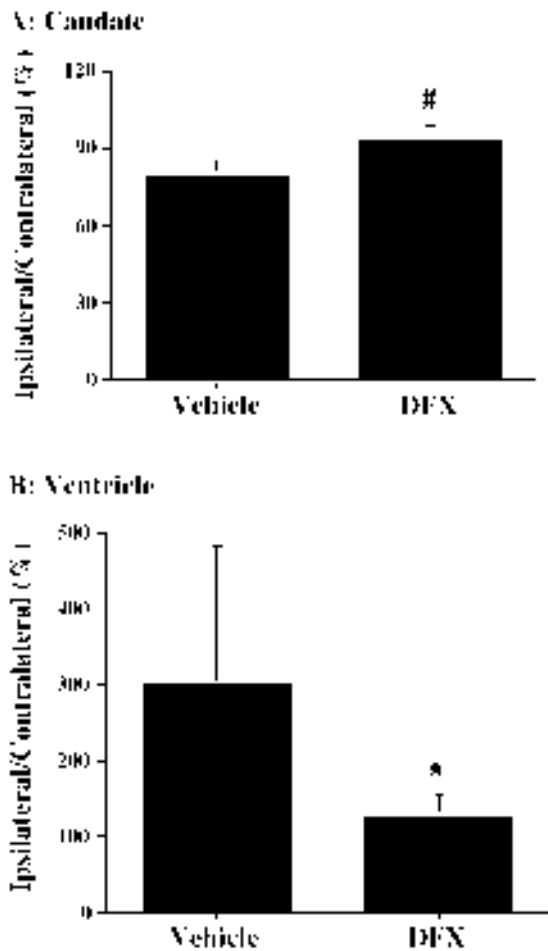


FIG. 5. Bar graphs showing results in deferoxamine- (DFX) compared with vehicle-treated rats in terms of attenuation of caudate atrophy (percentage of the contralateral caudate, A) and lateral ventricle enlargement (percentage of the contralateral lateral ventricle, B) evaluated 4 weeks after ICH. Values are the means \pm SDs, six rats per group (* $p < 0.05$ and # $p < 0.01$, compared with vehicle).

ICH (Fig. 4). All these results suggest that ICH-induced iron accumulation in the brain is not totally cleared after 12 weeks.

Deferoxamine treatment that was started 2 hours after ICH significantly attenuated caudate atrophy ($93 \pm 6\%$ compared with $79 \pm 6\%$ in the vehicle group; six rats; $p < 0.05$) and ventricle enlargement ($127 \pm 28\%$ compared with $300 \pm 181\%$ in the vehicle group; six rats; $p < 0.05$; Fig. 5). Forelimb placing and corner turn scores were also improved (Fig. 6). Nevertheless, there was only a trend toward improvement in the forelimb use asymmetry test. Deferoxamine also reduced ferritin-positive cells (Fig. 7) in the basal ganglia (57 ± 42 cells/mm² in the deferoxamine-treated compared with 240 ± 38 cells/mm² in the vehicle-treated group, $p < 0.01$) and motor cortex (39 ± 26 cells/mm² in the deferoxamine-treated compared with 212 ± 74 cells/mm² in the vehicle-treated group, $p < 0.01$).

Discussion

Clinical and experimental data have shown that brain

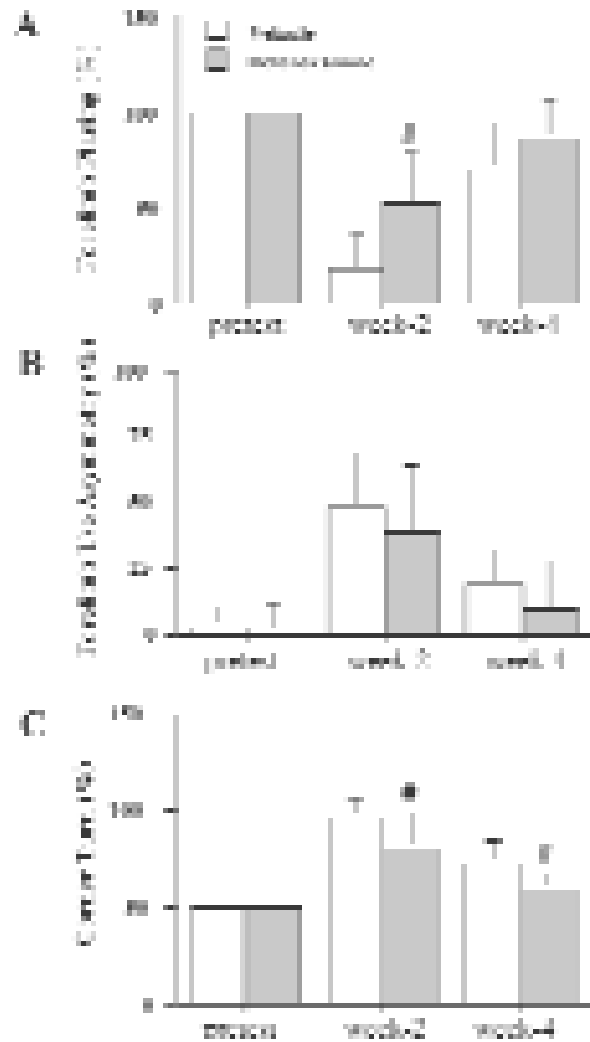


FIG. 6. Bar graphs displaying results of the forelimb placing, forelimb use asymmetry, and corner turn tests, which showed that deferoxamine reduced ICH-induced neurological deficits. Values are the means \pm SDs; six rats per group (# $p < 0.01$, compared with vehicle).

atrophy occurs after ICH.^{7,12,27,35} The underlying cause of this atrophy is, however, unknown. Data from our previous studies have indicated that hemoglobin and iron, its breakdown product, are major factors responsible for acute brain edema formation after ICH.^{13,20} Because iron has been implicated as a mediator in a number of neurodegenerative diseases,^{5,6} we focused on the role of iron in delayed brain injury.

In this study, we demonstrated that brain atrophy developed gradually and peaked between 1 and 2 months after ICH in the rat. Brain atrophy with prolonged neurological deficits was associated with iron accumulation in the brain tissue. Deferoxamine, an iron chelator, reduced brain atrophy and brain ferritin immunoreactivities, and improved neurological deficits. These results indicate that iron overload contributes to brain atrophy after ICH and that iron chelation may be a useful therapy for this condition.

Iron overload contributes to many kinds of brain injury, including Alzheimer disease and Parkinson disease.²⁹ Nev-

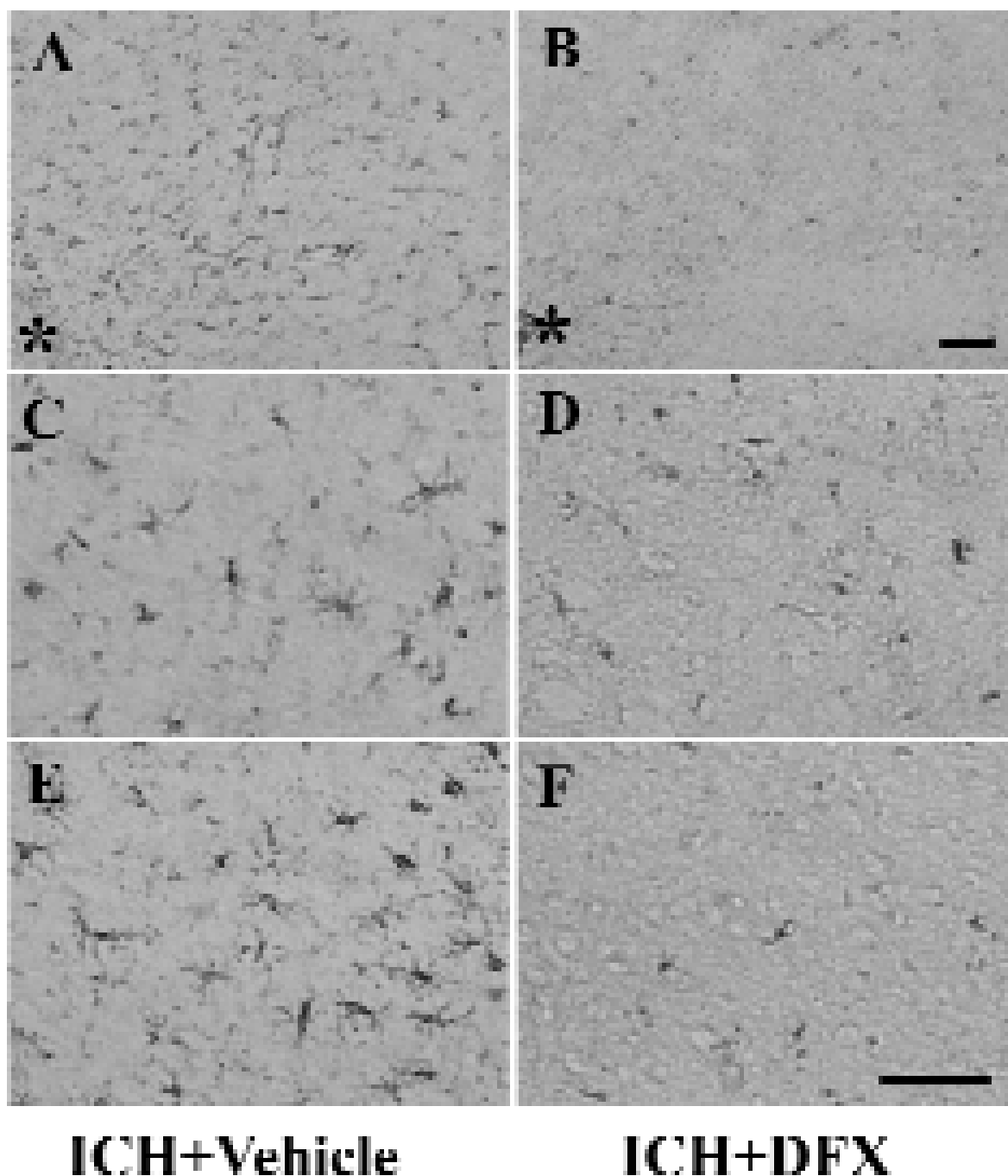


FIG. 7. Photomicrographs showing brain ferritin immunoreactivity in the ipsilateral basal ganglia (low power in A and B; high power in C and D) and the ipsilateral motor cortex (E and F) 4 weeks after induction of ICH and treatment with vehicle (A, C, and E) or deferoxamine (B, D, and F). Asterisks indicate the cavity of the hematoma. Bar = 100 μ m.

ertheless, the role of iron in ICH, one of the most severe diseases leading to abrupt iron overload, has not been well studied. After erythrocyte lysis, iron concentrations in the brain reach very high levels. Data from our recent studies revealed that there is a threefold increase of brain nonheme iron after ICH in rats and that levels remain high for at least 28 days.³³ In the present study, we show that iron remains in the brain, with a corresponding increase of ferritin immunoreactivity, for several months after ICH. Upregulation of

ferritin in the brain may be insufficient to prevent all iron-mediated damage after ICH. In addition, iron remains in the brain for a long time after ICH in humans.²⁸

The clot lyses and iron is released from heme within the 1st week after ICH, apparently contributing to acute brain edema formation.³⁶ Brain atrophy occurs several weeks later, however, suggesting that iron exposure causes cell damage that results in delayed cell death. For example, the iron may cause sufficient damage that the cell cannot repair it-

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self. If such death occurs in cells that are storing iron released from the hematoma, the new iron release may affect nearby cells, leading to amplification of the lesion. Alternatively, the iron stored in cells after resolution of the clot may be naturally released for clearance across the blood-brain barrier or through the cerebrospinal fluid, but with potential for causing further tissue damage.

Iron-induced brain damage may result from oxidation. Thus, iron catalyzes the conversion of $O_2 \cdot - H_2O_2$ to $\bullet OH - OH^- + O_2$ (the iron-catalyzed Haber-Weiss reaction; reviewed in Halliwell¹⁰), resulting in the production of the extremely reactive hydroxyl radical (that is, $\bullet OH$). Oxidative brain injury plays an important role in cerebral ischemia.^{2,3} Antioxidative treatments improve neurological deficits after ICH in a rat model of the disease induced by intracerebral injection of collagenase.²² Our recent data showing that lysed red blood cells increase protein carbonyl content in the brain indicate that erythrocyte lysis during clot resolution may be a major source of this oxidative stress.³⁴ Reactive oxygen species cause brain injury through many different pathways; one of these is direct DNA damage with resulting oxidative base damage and strand breaks. Such DNA damage has already been shown to occur in models of cerebral ischemia.^{4,19} Damage of DNA by reactive oxygen species can be greatly amplified in the presence of free iron,¹ and recent evidence supports the theory that oxidative stress is a major factor causing hemoglobin-induced cytotoxicity in vitro.³²

In a previous study, we found that there was a correlation between acute edema formation and early ICH-induced neurological deficits.¹¹ In the current long-term study, however, persistent neurological deficits were found even after the period when ICH-induced edema had resolved. Iron-induced brain damage may contribute to delayed neurological deficits. Sensorimotor impairments involving a loss of the capacity for sensory input to elicit an appropriate motor response are shared in both humans with ICH and in animal models of the disease. Rats are generally "front-wheel drive" (that is, when rats are held so that they support their weight only with the forelimbs, they can walk, but when they are held so that they support their weight only with the hindlimbs, they do not walk). Also, rats reach with their forelimbs, allowing examination of forelimb function, which can provide information that is relevant to both upper and lower extremities in humans. Study of vibrissae-evoked limb use in the rat is appropriate because, unlike humans, rats use primarily tactile information to guide movement. In contrast, the use of visual information by humans to guide them while walking is critical (visual input for humans and vibrissae input for rats are needed to ensure that a foot is placed on a stable surface). Determining how well sensory input can influence motor programs cuts across species regardless of the input used. When using the rat in a model, it is important to apply the most sensitive and reliable tests for the acute and chronic events associated with ICH.

Deferoxamine, an iron chelator, is approved by the Food and Drug Administration for the treatment of acute iron intoxication and chronic iron overload in transfusion-dependent anemias. Its molecular weight is 657, and deferoxamine can rapidly penetrate the blood-brain barrier and accumulate in brain tissue at a significant concentration after systemic administration.^{16,21} It is still controversial whether deferoxamine is effective in cerebral ischemia.^{8,18} It should

be noted, however, that the mechanisms of ischemic and hemorrhagic brain injury differ and that ischemia does not play an important role in our ICH model.³⁵ Our recent studies^{13,20} showed that deferoxamine reduces brain edema and early neurological deficits after ICH. In the present study we demonstrated that deferoxamine can reduce brain atrophy and improve behavioral outcome in a model of ICH in rats. These results support the theory that iron chelation may be a useful therapy for patients with ICH. It should be noted, however, that deferoxamine may protect the brain through noniron-chelation mechanisms. For example, deferoxamine can activate hypoxia-inducible factor-1 and induce ischemic brain tolerance;²³ it is also a free radical scavenger.^{14,18}

Conclusions

Brain atrophy with prolonged behavioral deficits occurred in rats in our model of ICH. The overload of iron in the brain after ICH is not cleared within 3 months. Treatment with deferoxamine reduces brain atrophy and improves functional outcomes in the rat after bleeding, indicating participation of iron in ICH-induced brain tissue loss, ventricular dilation, and neurological deficits. Deferoxamine may be a potential therapeutic agent for patients with ICH.

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