Conclusions—Rats with IDA have greater brain edema, poorer sensorimotor outcome, and a greater expression of iron regulatory proteins transferrin (Tf) and Tf receptor (TfR) respond in a way that suggests brain iron status is a determinant of injury severity and recovery. (Stroke. 2005;36:660-664.)

Key Words: anemia ■ behavior ■ intracerebral hemorrhage

Iron deficiency anemia (IDA) is a common nutritional disorder that occurs when body iron stores are not sufficient to meet the growth and maintenance needs of the individual. Insufficient dietary iron intake or significant iron losses increase the risk of IDA. Iron is important for many bodily processes, including hemoglobin production, oxidative metabolism, neurotransmitter synthesis, and myelination. IDA reduces brain iron concentration and alters behavior in rodents. Animal studies indicate that the iron regulatory proteins transferrin (Tf) and Tf receptor (TfR) respond in a heterogeneous fashion to iron deficiency and iron repletion and may regulate regional brain iron concentrations in response to local needs.

Intracerebral hemorrhage (ICH) is a common and often fatal subtype of stroke. If patients survive, the occurrence of hematoma within the brain parenchyma triggers a series of events that may lead to severe neurological deficits. Evidence suggests that iron released from hemoglobin and oxidative stress contribute to edema formation after ICH. In addition, the proteins involved in iron transport and storage (Tf, TfR, and ferritin) might modulate iron-induced cellular and neuronal toxicity, lipid peroxidation, and free radical formation.

The effects of iron accumulation on brain injury, Tf, and TfR levels after ICH have been investigated in nonanemic adult rats. We are not aware of studies evaluating the effects of IDA on ICH-related brain injury and recovery. Because iron can have neurotoxic effects, we hypothesized that outcome after ICH would be less severe with IDA because of less available iron. However, regional adaptations in brain iron regulatory proteins in IDA rats might predict increased brain iron uptake and thus a risk of more severe injury after ICH. To explore these questions, this study examined the effect of IDA on brain edema, functional behavior, and Tf and TfR expression after ICH in rats.

Materials and Methods

Animals and Experimental Groups
Seventy-five 6-week-old male Sprague-Dawley rats (Harlan, Indianapolis, Ind) were randomly assigned to 2 diet groups: non-IDA (iron-sufficient diet; 40 mg/kg iron) and IDA (iron-deficient diet; 3 to 6 mg/kg iron; Harlan Teklad). Both diets were adequate for protein and other micronutrients and were provided ad libitum for 1 month (28 to 31 days) before surgery to reduce hematocrit in the IDA group by ~40%. Food was weighed weekly. Animals continued their respective diets after surgery until brain assessment. All animals were pair-housed in a temperature-controlled (25°C) room with a 12-hour light/dark cycle. Approval for this protocol was provided by the University of Michigan committee for the care of animals.

Hematology
Blood was obtained weekly before surgery by tail vein puncture and assessed for hematocrit. Blood samples obtained at ICH surgery and...
ethanolsia were assessed for hematocrit and serum iron by standard methods.12

ICH Surgery
All animals were weighed then anesthetized with pentobarbital (40 mg/kg IP). Animals were placed on a feedback-controlled warming pad and rectal temperatures maintained at 36.5±0.5°C. Blood pressure, blood pH, arterial gases (PaO2, PaCO2), hematocrit, and glucose were obtained by femoral artery catheterization. Animals were positioned in a stereotactic frame (Kopf Instruments) and a 1-mm burr hole was drilled on the right coronal suture, 3.5 mm lateral to midline. A 26-gauge needle was placed in the right basal ganglia (BG): 0.2 mm anterior; 5.0 mm ventral; 3.5 mm lateral to bregma. A right BG hematoma was created by slowly infusing 100 μL autologous whole blood using a microinfusion pump (10 μL per minute; Harvard Apparatus). Six IDA rats received needle insertion into the right BG only (the IDA sham-operated group). After removal of the femoral artery catheter, incisions were closed with silk sutures, and the animals recovered before brain or behavioral assessments.

Brain Assessments
Regional brain edema was measured at day 3 after surgery for 5 to 6 animals in IDA and non-IDA ICH groups. Right and left BG and cortex were prepared for TIR Western blot analysis at days 1, 3, 7, and 28 after surgery for 3 to 4 animals in IDA and non-IDA ICH groups each day. Whole-brain non-heme iron concentration was determined for 6 animals from IDA and non-IDA groups using spectrophotometric methods as described previously.3

Brain Water Content
After deep anesthesia, animals were decapitated, whole brains removed, and a 3-mm-thick coronal brain slice was cut 4 mm from the frontal pole. Four samples were dissected from this slice: ipsilateral (injured) and contralateral (noninjured) BG and injured and noninjured cortex. Cerebellum was obtained as a control. Samples were weighed to 0.0001 g before and after drying for 24 hours at 100°C (gravity oven; Blue M. Electric). Brain water content was expressed as (wet weight−dry weight)/wet weight.

Behavioral Assessments
Upper extremity function was assessed with 2 sensorimotor tests before surgery and at days 3, 7 to 14, and 21 to 28 after ICH by an examiner (J.S.) blinded to animals’ diet group. Stimulation of vibrissae on 1 side normally results in same-side forelimb placement. Injury to BG or related sensorimotor tracks results in forelimb deficits contralateral to the injury.11,13,14 Vibrissa-stimulated forelimb placing was assessed by 10 trials for each forelimb and expressed as a percentage [right (injured BG side)−left (noninjured BG side)]. Forelimb use asymmetry was assessed by videotaping animals exploring a 20×30 cm plastic cylinder until 20 forelimb placements were observed. Forelimb use asymmetry was calculated as (right upper extremity+1/2 both)/total (right+left+both)×100.

Western Blot Analysis
Rats were reanesthetized, perfused with normal saline, and brains removed. A 3-mm coronal slice was cut 4 mm from the frontal pole. The slice was dissected into injured and noninjured BG. Regions were prepared for Western blotting as described previously.15 Brain tissue was immersed into 0.5 mL of Western sample buffer and sonicated. BG protein concentration was measured using Bio-Rad protein assay kits. Proteins (50 μg per sample) were run on 7.5% SDS-PAGE, then transferred to a Hybond-C pure nitrocellulose membrane (Amersham). Membranes were probed with 1:2500 polyclonal rabbit anti-human Tf (DakoCytomation, Carpinteria, Calif) or 1:500 monoclonal mouse anti-human TIR (Zymed, South San Francisco, Calif), followed by application of second antibody (1:2500 dilution; Bio-Rad, Hercules, Calif). Antigen-antibody complexes were visualized with a chemiluminescence system (Amersham) and exposed to photosensitive film. The relative density of each complex was analyzed using NIH Image software (version 1.61).

Statistical Analysis
Student t test and Mann–Whitney U test were used to compare hematologic, behavior, and brain variables by diet group or days after ICH. A heterogenous variance model was used to control for the effect of gel for Tf and TIR Western blot measurements to assess for differences by diet group. Diet group, day after ICH, and their interaction were assessed using Proc Mix. Pearson correlations for brain and behavior variables were explored. Significance was set at P<0.05.

Results
At ICH surgery, most physiological measures (blood gases, base excess, and blood glucose) were not significantly different (Table), and there was no mortality by diet group. Hematocrit, serum iron, blood pressure, and body weight were significantly reduced for IDA compared with non-IDA rats. IDA group rat food intake was 70% of non-IDA rats. IDA rat total non-heme iron brain was reduced by 41% compared with non-IDA rats (IDA 7.19±0.65 versus non-IDA 12.19±1.90; F=37.09; P<0.001).

Brain water content was significantly increased for the IDA-injured BG and cortex compared with non-IDA-injured BG and cortex (Figure 1). There was no significant difference by diet group for noninjured BG, cortex, or cerebellum (control).

Figure 1. Brain water content by diet group day 3 after ICH. Values are mean±SD; n=6; #P<0.05 vs non-IDA.
IDA and non-IDA ICH rats had significantly poorer vibrissae-stimulated left forelimb placing (contralateral to the injured BG; Figure 2A) than the IDA sham rats. This difference was observed throughout the month of assessment. Left vibrissae-stimulated forelimb placing was significantly impaired for IDA rats compared with non-IDA rats in the first 2 weeks after ICH. There was a trend for impaired forelimb placing at 1 month after ICH for IDA rats. Compared with non-IDA ICH rats, IDA ICH rats showed a trend for forelimb asymmetry at day 3 after ICH and day 3, n=20; days 7 to 14, n=12; days 21 to 28, n=6. n=8 for IDA sham.

Figure 2. A, Vibrissae-stimulated forelimb placing for the impaired limb. Values are mean±SD; #P<0.05 vs non-IDA ICH; ##P<0.01 vs IDA sham. B, Forelimb use asymmetry. Values are mean±SD; #P<0.05 vs non-IDA ICH (trend at day 3 P<0.06); ##P<0.02 vs IDA sham. For IDA and non-IDA ICH groups pre-ICH and day 3, n=20; days 7 to 14, n=12; days 21 to 28, n=6.

TF content in the injured BG peaked at days 1 to 3 for the IDA and non-IDA groups. However, TF was significantly increased in the injured BG for IDA rats compared with non-IDA rats on day 1 (Figure 3A) and day 7 after ICH. TF expression after ICH in IDA-injured BG was significantly increased at days 1 and 3 compared with days 7 and 28 (Figure 3B). Overall, there was a significant effect of diet group, day after ICH, and an interaction of diet group and day after ICH (Figure 3C). TF and TFR protein was assessed in noninjured BG for IDA and non-IDA rats. As shown in Figure 5, there was no significant difference for either protein in this region by diet group.

Correlations were explored for brain edema, behavior, and TF and TFR measures at day 3 after ICH. Injured BG water content was significantly correlated with asymmetric forelimb placing (Pearson correlation 0.629; P<0.05), and there was a trend for a negative correlation with forelimb placing response (Pearson correlation −0.510; P<0.10). Injured BG water content was also significantly correlated with injured BG TF expression (Pearson correlation 0.961; P<0.01) but not TFR expression in the injured BG (Pearson correlation 0.625; P=0.185).

Figure 3. BG TF by Western blotting. A, Day 1 after ICH. #P<0.05 vs other groups. B, Injured BG. IDA days 1 to 28 #P<0.05 vs days 7 and 28. C, Estimate of TF content by diet group and day after ICH. Diet and day, fixed effect; gel, random effect. Values are mean±SD. Diet group P<0.005; day P<0.001; diet group and day interaction P<0.03.
This study explores the effect of IDA on brain and behavioral outcome after ICH in rats. We demonstrate that IDA worsens brain injury severity and sensorimotor forelimb function recovery after ICH compared with those without IDA. Together, the findings suggest that IDA adversely affects brain injury and behavioral recovery after ICH in the rat and that the mechanisms may include edema formation and brain iron handling.

Neurological deficits after ICH are the consequence of brain edema, oxidative brain injury, and direct tissue destruction.\textsuperscript{7,11} Other factors could include the absence or delay of neurogenesis, resumption of normal function by ipsilateral neurons, or the assumption of new functions by other neurons (ipsilateral or contralateral). Brain edema after ICH is known to exacerbate brain injury, and the mechanisms of edema development have been investigated with interest in prevention or reduction. Several factors are now known to play key roles. Thrombin contributes to the early phase of brain edema within the first several hours after ICH.\textsuperscript{15,16} Delayed edema formation occurs by \textasciitilde3 days after ICH and involves erythrocyte lyses and cellular toxicity attributable to hemoglobin and its degradation products.\textsuperscript{17}

The behavioral findings in this study suggest that greater or longer-lasting brain edema and consequent space-occupying effects may be a factor contributing to the poorer behavioral outcome for IDA rats. This prediction is based on the correlations of behavioral function and degree of brain edema at day 3 after ICH in the present study and our previous study, which demonstrated that behavioral outcome after ICH correlates with the degree of brain edema over time after ICH.\textsuperscript{11} In that study of nonanemic adult rats, brain edema resolved and behavioral recovery stabilized within 2 weeks after ICH. However, in the present study, IDA rats had poorer vibrissae-stimulated forelimb placing than nonanemic rats during the first 2 weeks and continued to have significantly poorer forelimb asymmetry at 3 to 4 weeks after ICH. This suggests greater initial or longer-lasting edema after ICH with IDA.

Because the IDA hematocrit was 50% less than the nonanemic group, one might have expected brain edema to be reduced at day 3 after ICH for the IDA rats on the basis of less hemoglobin and degradation products known to contribute to delayed edema formation.\textsuperscript{11} However, brain edema was greater for IDA rats. A possible mechanism for this finding relates to the increased serum volume (\textasciitilde33%) in the IDA infusate. If the absolute amount of thrombin delivered in the IDA infusate was greater, this could contribute to the early phase of edema formation. Hua et al demonstrated previously that thrombin worsens brain edema and behavioral outcome and that hirudin, a thrombin inhibitor, improves these measures after ICH in the nonanemic adult rat.\textsuperscript{11} In addition, overexpression of interleukin-1 receptor antagonist by microglia attenuates brain edema induced by ICH and thrombin.\textsuperscript{19} To clarify the mechanisms underlying greater edema and poorer behavioral outcome with IDA, several assessments would be fruitful, including: measuring edema formation over time after ICH with IDA; measuring the concentration of prothrombin in IDA sera; and investigating whether thrombin inhibitors reduce edema formation and improve behavioral outcome in IDA rats after ICH.

Previous studies in nonanemic adult rats indicate that local iron overload may play a role in brain injury after ICH. Relative,
regional iron overload appears to contribute to brain injury via lipid peroxidation pathways and the formation of free radicals. A recent study reported a 3-fold increase in brain non-heme iron in the perihematoma zone after ICH in rats. Oxidative brain injury after ICH has been confirmed, and antioxidants block neuronal toxicity induced by hemoglobin and iron. In the present study, one might have predicted less brain injury and improved behavioral outcome on the basis of lower total non-heme brain iron in IDA rats at baseline and the likelihood of lower iron delivered in the infusate (supported by lower hematocrit and serum iron). However, we found greater brain edema and poorer behavioral outcome, suggesting it may not be the absolute amount of iron, but rather how iron is managed in the IDA brain after ICH.

We hypothesize that although greater Tf and TfR expression after ICH may be adaptive in noninjured IDA rats, altered brain iron trafficking in the context of IDA may contribute to exaggerated brain injury. Tf and TfR are involved in iron transport and are substantially increased in the injury area after ICH in the nonanemic rat. Tf has been demonstrated in oligodendrocytes, and TfR appears localized to neuron-like cells in perihematoma area after ICH. It has been hypothesized that increased iron trafficking proteins are related to clearance of locally high iron concentrations in the injured area after ICH. In the context of iron deficiency, whole-brain Tf concentration is increased, but there is considerable regional variability for Tf and TfR expression. Striatal Tf levels appear to be relatively preserved compared with other brain regions in the context of IDA. In the present study, Tf and TfR were increased in the perihematoma area for IDA rats compared with nonanemic rats at days 1 to 3 after ICH, and TfR remained significantly elevated for IDA rats ≤1 month after ICH. Because baseline Tf and TfR protein levels in the BG did not differ as a consequence of IDA (without ICH), our findings suggest that degree and duration of brain iron handling protein response after ICH may be greater in the iron-deficient brain. Future studies might explore whether greater oxidative injury occurs as a consequence of local iron overload and the response of iron trafficking proteins after ICH in the context of IDA.

In this study, we explored the effect of IDA on ICH on brain and behavioral outcome. However, there were important limitations. The IDA group gained less weight than the nonanemic rats. Although this observation is consistent with other IDA studies, thought attributable in part to less food intake during a period when the rats were still growing, future studies of ICH in the context of IDA should include a pair-fed control group to control for potential unmeasured factors related to iron deficiency. This study assessed brain edema at day 3 after ICH to capture early and delayed edema formation. Our behavioral findings suggest it would be fruitful to assess the course of edema after ICH in the context of IDA. It would also be important to document the cell types expressing Tf and TfR in the perihematoma area and adjacent regions and investigate ferritin expression and localization to clarify how IDA influences the handling of iron after relatively iron-rich injury associated with ICH. The study by Wu et al suggests that after ICH, iron concentrations are greater in the injured versus the noninjured hemisphere after ICH. It would be interesting to explore this after ICH with IDA.

In conclusion, IDA rats have more severe brain edema and poorer sensorimotor outcome after ICH than non-IDA rats, suggesting that iron status at the time of brain insult is an important determinant of injury severity and recovery. These results are consistent with a study by Rao et al, which demonstrated worse outcome after hypoxia ischemia in the context of IDA. To apply appropriate therapeutic decisions for iron-deficient anemic individuals with ICH, further study is needed to understand the mechanisms unique to ICH with IDA.

References

Intracerebral Hemorrhage in the Iron-Deficient Rat
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