Low Molecular Weight Iron in Cerebral Ischemic Acidosis In Vivo

Diane C. Lipscomb, MD; Linda G. Gorman, PhD; Richard J. Traystman, PhD; Patricia D. Hurn, PhD

Background and Purpose—Iron-catalyzed radical generation is a potentially significant mechanism by which extensive tissue acidosis exacerbates brain injury during ischemia/reperfusion. We hypothesized that levels of low-molecular-weight (LMW) iron increase during in vivo global cerebral ischemia in a pH-dependent manner, potentially catalyzing oxidant injury. The present study quantified regional differences in LMW iron during global cerebral incomplete ischemia and determined whether augmenting the fall in ischemic tissue pH with hyperglycemia also amplifies free iron availability.

Methods—Dogs anesthetized with pentobarbital-fentanyl were treated with 30 minutes of global incomplete cerebral ischemia produced by intracranial pressure elevation. Cerebral energy metabolites (ATP, phosphocreatine) and intracellular pH (pHi) were measured by 31P magnetic resonance spectroscopy. Preischemic plasma glucose level was manipulated to titrate end-ischemic pHi. After ischemia, brains were perfused with cold phosphate-buffered saline solution; then 16 different brain areas were sampled, filtered to separate the LMW fraction (30 000 D), and assayed by rapid colorimetric assay for tissue iron. Total iron, LMW iron, and protein in each sample were measured in sham-operated (no ischemia, n = 8), normoglycemic ischemia (ISCH [glucose 7 ± 6 mmol/L], n = 7), and hyperglycemic (GLU-ISCH [glucose 31 ± 6 mmol/L], n = 9) groups.

Results—High-energy phosphates fell to near zero values in both ISCH and GLU-ISCH groups by 30 minutes but remained unchanged in the sham-operated group. As expected, pH decreased during ischemia but to a greater extent in GLU-ISCH (6.20 ± 0.05 in ISCH, 6.08 ± 0.04 in GLU-ISCH, P < .05). Iron could be detected in all areas of the brain in sham-operated animals, with the highest amounts obtained from subcortical areas such as the hippocampus, pons, midbrain, and medulla. Total iron was higher in ISCH relative to sham-operated animals and higher in cortex and pons relative to GLU-ISCH. Regional LMW (as a percentage of total iron; LMW/total iron) was elevated in numerous brain areas in ISCH, including cortical gray matter, cerebellum, hippocampus, caudate, and midbrain. LMW/total iron was higher in GLU-ISCH versus ISCH in cortical gray matter only. In other brain areas, ischemic LMW/total iron was equivalent in glucose-treated or normoglycemic animals (white matter, thalamus, pons, medulla) or lower in the glucose-treated group (cerebellum, hippocampus, caudate, midbrain).

Conclusions—These data demonstrate that levels of total and LMW iron increase with global cerebral ischemia in the majority of cortical and subcortical regions of normoglycemic brain. However, exacerbation of ischemic acidosis via glucose administration does not increase tissue iron and produces a greater increase in the LMW fraction in cortical gray matter only. In other brain regions, total and LMW iron availability is similar to that of nonischemic animals. (Stroke. 1998;29:487-493.)

Key Words: acidosis • iron • cerebral ischemia • spectroscopy, nuclear magnetic resonance

Iron-catalyzed oxygen radical production has long been hypothesized to play a prominent role in brain injury during ischemia/reperfusion. Via Fenton-Haber chemistry, ferrous iron (Fe2+) interacts with hydrogen peroxide or superoxide to produce hydroxyl radical and other free radicals that damage cell protein and DNA structure.1-3 Alternatively, iron is an effective catalyst of tyrosine nitration by another cytotoxic oxidant, peroxynitrite.4 Furthermore, radical-mediated mechanisms may be particularly relevant to cerebral injury when the ischemic insult is accompanied by extensive tissue acidosis, eg, during ischemia in hyperglycemic subjects. Rehncrona et al5 hypothesized that lactic acidosis increases iron dissociation from carrier proteins that use carbonate binding, whereas carbonic acidosis (eg, from tissue CO2) acts to stabilize bicarbon-
plays an important role in acidosis-mediated mechanisms of ischemic injury, the conditions of iron release, regional distribution of prooxidant iron, and participation in lipid peroxidation have not been clearly outlined.

We have previously shown that reducing intracellular pH (pH) to <6.0 and bicarbonate ion to <1 to 2 mmol/L during hyperglycemic ischemic cerebral ischemia produces a marked secondary deterioration of brain ATP and cerebral blood flow during reperfusion.9 This rapid and profound secondary injury can be ameliorated with antioxidant treatments, including the iron chelator and superoxide scavenger, deferoxamine.10,11 Therefore, we wished to further examine the role of iron in ischemic injury and quantify the increase in nonprotein bound iron that ordinarily occurs during global cerebral ischemia in vivo. The goal of the present study was to evaluate regional distribution of cerebral iron during ischemic conditions compared with the nonischemic brain. Furthermore, we tested the hypothesis that reduction of tissue pH during hyperglycemic ischemia amplifies free iron, some component of which may participate in oxidant-mediated brain injury.

### Materials and Methods

This study was approved by the institutional animal care and use committee and is in compliance with the guidelines of the National Institutes of Health for care and handling of animals. A well-characterized model of global incomplete cerebral ischemia in dogs was used.2,5 Male dogs (10 to 15 kg) were anesthetized with intravenous fentanyl (50 μg/kg) and pentobarbital (6 mg/kg, followed by 3 mg/kg per hour) and mechanically ventilated with supplemental oxygen. The dogs were paralyzed with pancuronium bromide (0.1 to 0.2 mg/kg). Systemic arterial and venous catheters were placed for blood pressure monitoring, blood gas sampling, and infusion of intravenous fluids and medications. After complete retraction of temporalis muscles, a midline burr hole was placed near the junction of the coronal sutures for placement of a superior sagittal sinus. A Silastic ventricular drain catheter (Cordis) was placed into the ventricle via a second burr hole for intracranial pressure monitoring and infusion of artificial cerebrospinal fluid. An epidural thermometer was placed for continuous temperature monitoring. To maintain normothermic epidural temperature, animals were warmed as needed with a water blanket and head insulation.

Arterial and sagittal sinus blood samples were analyzed for PO2, P[scap]co2, and pH levels with a Radiometer ABL electrode system. Oxygen content was measured with a CO-Oximeter (No. 282, Instrumentation Laboratories). Arterial blood pressure and intracranial pressure were measured with Statham transducers. Serum glucose levels were measured on a YSI model 23A Glucose Analyzer. Using a Vivospec spectrometer (Otsuka Electronics with a 1.89-T horizontal superconducting magnet, 25-cm bore; Oxford Instruments),31P MRS spectra were obtained as previously described.9,10,12 Spectral areas were analyzed via planimetry for B-ATP and phosphocreatine and expressed as a percentage of the respective baseline areas. pHi was determined by methods previously described by Petroff et al.,9 using constants derived from our titration data:

\[ \text{pHi} = -6.73 + \log[a(-3.07)/5.68 - a] \]

where \( a \) is the chemical shift of inorganic phosphate relative to phosphocreatine. An external standard (dimethyl[2-oxopropyl]-phosphonate) placed over the coil served as a marker for spectral position when the phosphocreatine peak disappeared. The Henderson-Hasselbalch equation was used to calculate intracellular [HCO3 -] using a pK of 6.12, pH as measured by MRS, and sagittal sinus PCO2 with a solubility coefficient of 0.0314 mmol/L per mm Hg. Differences in sagittal sinus P[scap]co2 were assumed to reflect changes in tissue P[scap]co2.

The dogs were divided into three treatment groups: sham-operated without ischemia, normoglycemic (ISCH), or hyperglycemic ischemia (GLU-ISCH). In GLU-ISCH, plasma glucose was raised to ≈560 mg/dl with intravenous 50% dextrose solution immediately before and during ischemia. Baseline measurements of arterial and venous blood gases, intracranial pressure, blood pressure, glucose, and MRS spectra were obtained in all groups. To create global incomplete cerebral ischemia, warmed cerebrospinal fluid was infused into the lateral ventricle from a reservoir, raising intracranial pressure and lowering cerebral perfusion pressure to a controlled level of 10 to 15 mm Hg, yielding a low residual cerebral blood flow. The ischemic period was 30 minutes.9–12 MRS data were collected throughout the experimental period in one 6-minute epoch and three 8-minute epochs. To end ischemia, the reservoir was disconnected and intracranial pressure rapidly returned toward baseline. The animal was removed from the magnet, and a left lateral thoracotomy was performed with ventricular cannulation and perfusion of 1 L of phosphate-buffered 0.9% NaCl solution to clear blood from the cerebral vasculature. The skull cap was removed at 15 minutes of reperfusion in each animal, and 16 different brain areas were sampled and frozen in liquid nitrogen for neurochemical analysis.

Brain samples were evaluated for levels of LMW and total iron by the rapid colorimetric method as described by Fish.14 All assay solutions were made from double-chelated water to minimize iron contaminants (Chelex-100, BioRad). All chemicals were purchased from Sigma Chemical Co. Samples were weighed and then homogenized in 1-mmol/L EDTA to a final concentration of 40 mg tissue/mL and 1-mmol/L EDTA. An aliquot was obtained for subsequent analysis of total iron and protein. The LMW aliquot was centrifuged in a three-step process: (1) at 3000 rpm for 20 minutes, (2) at 19 000 rpm for 40 minutes, and (3) the supernatant was then spun at 6000 rpm for 60 minutes through a 30 000 molecular weight nylon filter collection system (Vanex VG8504–00, Vanguard International) to separate the LMW fraction. Total and LMW iron samples were assayed by incubating 400-μL aliquots with 200 μL 1.2 mol/L hydrochloric acid–0.285 mol/L potassium permanganate solution for 2 hours at 60°C to release tissue iron. Next, 40 μL of 2 mol/L ascorbic acid–5 mol/L ammonium acetate–13.1 mol/L ferrozine solution was added to chelate ferric iron species and other potentially interfering metals. To precipitate iron and protein precipitates, total iron samples were centrifuged for 1 minute on an Allied-Fisher Scientific Micro-Centrifuge model 235C. A standard curve was generated for the range of 0.01 to 2.0 μg/mL, and all samples were measured at 562 nm with a Milton Roy Spectronic 601 spectrophotometer. Total protein levels were determined using the Bradford method.

Because minimal differences were apparent in the eight cortical regions, the data are presented as summed gray matter and individually represented deep brain structures. Data are normalized to total iron within the sample and to the sample wet weight. To evaluate reproducibility and assess intra-animal variability, a brain region from one sham-operated, one ISCH, and one GLU-ISCH animal each was homogenized, and several samples from the same region per animal were assayed using study methods. The coefficient of variation for this repeated analysis was 3.6±0.0%. To these known samples, predetermined amounts of iron were added. Measurements were accurate to within 0.06 μg iron.

Because cerebral blood flow was not measured in the present study, severity of ischemia was assessed by the lack of any residual phosphocreatine signal and severe depression of ATP by end-ischemia as previously observed with this ischemic model and duration.9–12 Therefore, any animal with measurable phosphocreatine (>5%) by 30 minutes of ischemia was excluded from the study. Eight animals were excluded on this basis (5 normoglycemic and 3 hyperglycemic dogs).
The data are presented as mean±SEM, and significance was set at $P<.05$ for all tests. Physiological data were analyzed with a repeated measures ANOVA, with treatment as the between-subject factor and time as the within-subject factor. If group or time interactions were significant, a Newman-Keuls test was used to distinguish individual groups at specific time points. Neurochemical data were analyzed using a one-way ANOVA and Newman-Keuls test.

### Results

Physiological data are contained in Table 1. Arterial blood gases were controlled to maintain constant oxygenation and arterial CO$_2$; however, arterial pH was depressed in both ischemic groups relative to sham-operated animals. Epidural temperature decreased in both ischemic groups compared with sham but in an equivalent manner. During ischemia, arterial blood pressure initially rose in ISCH and GLU-ISCH, then decreased to baseline levels. Cerebral perfusion pressure was maintained at approximately 10 mm Hg, and there were no differences between the ischemic groups. Cerebral perfusion pressure and intracranial pressure remained unchanged over time in the sham-operated group. Plasma glucose was elevated throughout ischemia in GLU-ISCH relative to both ISCH and sham groups. During ischemia, hemoglobin was lower than in the sham-operated and ISCH groups.

As previously shown in this model, high-energy phosphates declined sharply during the 30-minute period of intracranial pressure elevation (Fig 1). At end-ischemia, phosphocreatine and ATP levels were similar between ISCH and GLU-ISCH. As expected, pH, decreased in both ischemic groups but to a greater extent in GLU-ISCH (Fig 2). End-ischemic pH, was 6.20±0.05 in ISCH and 6.08±0.04 in GLU-ISCH ($P<.05$).

Levels of total and LMW iron were determined in 16 different brain regions. Because minimal differences were apparent in the eight cortical regions, the data are presented as summed gray matter (Table 2). Iron could be detected in all areas of the brain in sham-operated animals, with the highest amounts obtained from subcortical areas such as the hippocampus, pons, midbrain, and medulla. Total iron was higher per gram of wet weight in ISCH animals than in the sham group in most brain areas and higher in ISCH than in GLU-ISCH in three brain regions. In gray matter, LMW iron was 3.06±1.09


The role of iron in oxidant injury as a consequence of ischemia has been inferred from numerous studies that show benefit from treatment with strong iron chelators or from aprotinin. We found previously that pretreatment with deferoxamine, but not conjugated deferoxamine or iron-saturated feroxamine, ameliorates the secondary metabolic deterioration and severe hyperperfusion observed with ischemia complicated by extreme lactic acidosis. However, deferoxamine has significant radical scavenging properties in vitro via hydrogen-donating free hydroxamate groups, so its mechanism of action relative to iron chelation is not entirely clear. Oxidative injury has been ameliorated by inhibiting iron-dependent lipid peroxidation with aminosteroids after trauma and ischemia.

Consequently, whether ischemia-induced release of substantial amounts of reactive iron occurs in vivo is of interest. LMW ferrous iron has been demonstrated to increase at various reperfusion time points after cardiac arrest, with the large increases observed at 1 to 2 hours after arrest, and to be accompanied by increases in levels of lipid peroxidation. Our results with incomplete global cerebral ischemia are consistent with these earlier findings in cortical homogenates, and we now show that increased free iron availability is widespread in brain, including regions of the brain that are known to be highly vulnerable to ischemic insults. Measurable levels of LMW iron were present in the nonischemic brain, with the greatest levels of iron in the white matter of all groups. Others have demonstrated iron concentration in white matter, midbrain, and we now show that increased free iron availability is widespread in brain, including regions of the brain that are known to be highly vulnerable to ischemic insults.

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which is a major source of total tissue iron. It seemed likely that pH-associated delocalization of iron would be through release and reuptake of iron from carrier/storage proteins. Nevertheless, our measurements do not determine the precise chemical identity or catalytic activity of the LMW iron; small iron-complexed moieties and heme-containing proteins less than 30 000 D are likely present as LMW species and potential nonparticipants in iron-catalyzed oxidative injury.

The mechanisms of iron release during ischemia are likely complex, potentially involving both intracellular stores and intravascular iron transport and delivery to brain. Because of the potential cytotoxicity of iron, physiological systems have developed intricate mechanisms to protect cells from oxidative injury. Brain iron is largely complexed to protein carriers (transferrin, lactoferrin) for intravascular and transmembrane transport and to ferritin for intracellular storage. Ischemic acidosis may drive iron dissociation from these carrier proteins with pH-dependent binding characteristics (eg, carbonate binding ion of transferrin).24 Once mobilized, free iron likely binds nonspecifically to a variety of small molecular moieties and augments the ordinarily small LMW nonprotein-bound tissue pool. In cortical homogenates, striking increases in LMW iron are observed at pH 6.0 when pH is reduced from 7.0 by direct addition of lactic acid. Furthermore, brain from decapitated hyperglycemic rats shows elevated LMW iron relative to normoglycemic controls.7

Surprisingly, we did not observe increased LMW iron in all brain regions in hyperglycemic ischemia despite reduction in pH, to values <6.0 in many glucose-treated animals. Total iron was higher in many but not all brain regions in ISCH; consequently, we evaluated regional LMW iron for each group as a percentage of total iron. LMW/total iron was elevated in hyperglycemic versus normoglycemic ischemia only in cortical gray matter. In other brain areas, ischemic LMW/total iron was equivalent or lower in brain of glucose-treated animals when compared with either normoglycemic or nonischemic brain, suggesting that lower end-ischemic pH, does not exacerbate ischemia-induced iron availability for catalysis of oxidant injury mechanisms, at least within the one time-point window of our observations. We cannot exclude the possibility that lower pH in GLU-ISCH accelerated iron dissociation, peaking during ischemia or immediate reperfusion with subsequent early elimination of the LMW tissue pool. In addition, the end-ischemic pH, achieved in ISCH and GLU-ISCH spanned a narrow range. We did not evaluate iron over a range of pH to determine an optimum pH associated with LMW iron augmentation or whether there is a threshold below which free iron begins to decrease because of other pH-related factors. Nevertheless, it seems unlikely that the large differences observed with ISCH versus GLU-ISCH are explained by these factors. A more plausible explanation is that the total iron pool, and consequently the LMW fraction, increases during ischemia/reperfusion in an unanticipated manner that is independent of pH depression. However, with hyperglycemic ischemia, total tissue iron and the LMW fraction are regionally heterogeneous and not uniformly higher than in nonischemic animals.

One hypothesis is that ischemic brain iron loads are linked to intravascular iron delivery and tissue transfer via normal or pathophysiological mechanisms. The ischemic insult, as determined by the near complete loss of ATP and phosphocreatine, was different between ischemic groups in plasma glucose level only (7 versus 31 mmol/L in ISCH and GLU-ISCH, respectively) and the severity of end-ischemic pH (6.20 versus 6.08). Our previous measurements with this model indicate that brain blood flow, as measured by radiolabeled microspheres, is reduced uniformly throughout the brain, with a residual flow of <10 to 12 mL/min per 100 g during both normoglycemic and hyperglycemic ischemia.9–12 However, we sampled tissue after 30 minutes of ischemia and 15 minutes of reperfusion in each animal. Postischemic hyperemia occurs in normoglycemic animals during early reperfusion9–12; hyperglycemic ischemia results in heterogeneous hyperemia9 and significant hypoperfusion.7,11,12,26,27 While it is uncertain that extensive hypoperfusion would account for the failure to see increased total and LMW iron in the GLU-ISCH group, de novo iron delivery from circulating iron containing proteins could be reduced during hyperglycemic reperfusion. Although the kinetic and mechanisms of iron transport across the postischemic blood-brain barrier are not known, iron transfer into cells via transferrin binding and endosomal internalization is thought to occur within minutes in healthy brain.6 If intravascular iron delivery to tissue represents a significant contribution

### TABLE 2. Iron Levels During Global Incomplete Ischemia

<table>
<thead>
<tr>
<th>Area</th>
<th>Total Iron/Weight, μg/g</th>
<th>LMW Iron/Total Iron, % of Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>ISCH</td>
</tr>
<tr>
<td>Gray matter</td>
<td>1.56±0.23</td>
<td>4.41±1.33†</td>
</tr>
<tr>
<td>White matter</td>
<td>0.55±0.14</td>
<td>2.92±0.95†</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>0.09±0.03</td>
<td>0.11±0.03</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.40±0.04</td>
<td>1.17±0.31*</td>
</tr>
<tr>
<td>Caudate</td>
<td>0.52±0.06</td>
<td>0.38±0.25</td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.71±0.14</td>
<td>2.58±0.99*</td>
</tr>
<tr>
<td>Midbrain</td>
<td>0.32±0.07</td>
<td>0.83±0.26*</td>
</tr>
<tr>
<td>Pons</td>
<td>0.46±0.10</td>
<td>4.98±2.03†</td>
</tr>
<tr>
<td>Medulla</td>
<td>0.25±0.11</td>
<td>0.99±0.32*</td>
</tr>
</tbody>
</table>

All data are expressed as mean±SEM. Sham-operated (n=8); ISCH (n=9); GLU-ISCH (n=7). *P<.05 from sham group; †P<.05 from GLU-ISCH.
to the dynamic pool in reperfused brain, then gross hyperperfusion would alter tissue iron content.

An alternative hypothesis is that the low levels of iron in GLU-ISCH are a function of the high plasma and tissue glucose levels per se. Hyperglycemia glycates many protein moieties, including transferrin and increases tissue autooxidation products. Transferin-bound iron is low in rat hyperglycemic sera secondary to glycation, although the proteins remain redox-active, and target cell binding is depressed. Therefore, this source of iron dissociation may be inconsequential in GLU-ISCH versus ISCH. The effect of acute hyperglycemia on intracellular storage proteins such as ferritin is unknown; however, with intense oxidant stress, ferritin can be transformed into a hemosiderin-like protein, which is less reactive to Fe\(^{2+}\)-releasing stimuli. The abnormal ferritin-Fe\(^{2+}\) complex would not be captured in our fractionation assay because of its relatively high molecular weight. Therefore, it is feasible that hyperglycemia alters carrier protein binding in normal or ischemic tissue and yields lower brain iron during reperfusion. Our present method for quantifying the total or LMW iron pool is not sufficiently sensitive to distinguish a baseline effect of hyperglycemia in sham-operated animals, LMW iron pool is not sufficiently sensitive to distinguish a baseline effect of hyperglycemia in sham-operated animals, independent of ischemia.

In conclusion, our findings strengthen the hypothesis that tissue iron and its potentially reactive LMW fraction are elevated after global incomplete cerebral ischemia and now demonstrate that the regional distribution is widespread. However, when the fall in tissue pH ordinarily observed with ischemia is exacerbated by systemic hyperglycemia, total iron and subsequently the LMW iron species available for catalysis of oxidant injury mechanisms are not elevated during early reperfusion. Whether other means of altering ischemic pH produce a similar lack of ischemia-induced iron release remains to be tested. However, we speculate that hyperglycemia alters ischemic tissue iron content in a pH-independent manner, possibly by decreasing iron delivery, tissue internalization, and binding protein function during severe but incomplete cerebral ischemia and early reperfusion.

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References

There is extensive evidence that hyperglycemia exacerbates cerebral ischemic injury. Whereas hyperglycemic patients have a worse neurological outcome after ischemic stroke, preischemic hyperglycemia aggravates ischemic brain damage in experimental animals.1,2 The mechanisms of this effect have not been elucidated. It has been suggested that the worsening of the brain damage is secondary to the greater pH reduction associated with hyperglycemic ischemia.3 More severe acidosis would in turn increase in the availability of free iron for formation of reactive oxygen species through the Haber-Weiss reaction. In the accompanying article, Lipscomb et al sought to test this hypothesis in vivo using a well-established canine model of temporary global cerebral ischemia. As anticipated, hyperglycemic ischemia produced a more severe reduction in brain pH, than normoglycemic ischemia. Furthermore, normoglycemic ischemia elevated LMW iron in most brain regions. Unexpectedly, however, hyperglycemic ischemia enhanced this effect only in the neocortical gray matter and not in other brain regions. Rather, in most regions, ischemic hyperglycemia resulted in iron concentrations similar to those observed in normal brain. The data suggest that in this model, increased iron availability does not play a prominent role in the exacerbation of brain damage associated with hyperglycemic ischemia, with the possible exception of the cerebral cortex.

The regulation of iron homeostasis in the postischemic brain is poorly understood. Iron, a metal insoluble in water at physiological pH, is transported in the blood stream as Fe^{3+}-bound transferrin (see Reference 4 for review). The transferrin-iron complex enters the cell by endocytosis via membrane-bound transferrin receptors. Once inside the cell, iron is sequestered by the storage protein ferritin from which it is released as Fe^{2+} (see Reference 5 for review). Expression of critical proteins involved in iron metabolism, eg, ferritin H and L chains, the transferrin receptor, and 5-aminolevulinate synthetase (the enzyme that synthesizes heme), is controlled by the iron response element–binding protein (IRE-BP).5 Thus, the intracellular concentration of catalytically active iron is tightly regulated by these proteins, the expression of which is controlled by IRE-BP. The effect of cerebral ischemia and/or hyperglycemia on this complex sequence of events remains to be elucidated. The work of Lipscomb et al is a step in the right direction because it provides much awaited in vivo data on the postischemic perturbation in iron homeostasis and on the effect of hyperglycemia on iron availability. This enlightening study constitutes a starting point for future investigations focusing on the effect of cerebral ischemia on the cellular and molecular factors regulating free intracellular iron.

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