Acute and Delayed Deferoxamine Treatment Attenuates Long-Term Sequelae After Germinal Matrix Hemorrhage in Neonatal Rats

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Background and Purpose—This study investigated if acute and delayed deferoxamine treatment attenuates long-term sequelae after germinal matrix hemorrhage (GMH).

Methods—Bacterial collagenase (0.3 U) was infused intraparenchymally into the right hemispheric ganglionic eminence in P7 rat pups to induce GMH. GMH animals received either deferoxamine or vehicle twice a day for 7 consecutive days. Deferoxamine administration was initiated at either 1 hour or 72 hours post-GMH. Long-term neurocognitive deficits and motor coordination were assessed using Morris water maze, rotarod, and foot fault tests between day 21 to 28 post-GMH. At 28 days post-GMH, brain morphology was assessed and extracellular matrix protein (fibronectin and vitronectin) expression was determined.

Results—Acute and delayed deferoxamine treatment improved long-term motor and cognitive function at 21 to 28 days post-GMH. Attenuated neurofunction was paralleled with improved overall brain morphology at 28 days post-GMH, reducing white matter loss, basal ganglia loss, posthemorrhagic ventricular dilation, and cortical loss. GMH resulted in significantly increased expression of fibronectin and vitronectin, which was reversed by acute and delayed deferoxamine treatment.

Conclusions—Acute and delayed deferoxamine administration ameliorated long-term sequelae after GMH. (Stroke. 2014;45:00-00.)

Key Words: deferoxamine extracellular matrix proteins

Germinal matrix hemorrhage (GMH) occurs when immature blood vessels rupture within the subventricular tissue in premature infants. GMH occurs in ≈12,000 live births per year in the United States and often results in developmental delays, mental retardation, cerebral palsy, and posthemorrhagic hydrocephalus, posing significant socioeconomic burdens. Because current clinical management is limited, research is needed to investigate innovative therapeutic modalities.

In adult rodent intracerebral hemorrhage and subarachnoid hemorrhage models, red blood cells present in the intraparenchymal tissue are lysed and hemoglobin metabolized to release iron, resulting in a highly oxidative environment and consequent iron toxicity that damages brain tissue. Chelation of iron using deferoxamine improved neurofunctional outcomes after intracerebral hemorrhage and subarachnoid hemorrhage, yet its efficacy has not been evaluated after neonatal GMH. Blood products and proliferation of extracellular matrix proteins are theorized to disrupt cerebrospinal fluid flow dynamics after hemorrhage, leading to consequent posthemorrhagic hydrocephalus development.

We hypothesized that acute and delayed deferoxamine treatment will ameliorate extracellular matrix protein proliferation, posthemorrhagic ventricular dilation, and long-term neurofunctional outcomes after GMH.

Methods All protocols and procedures were approved by the Institutional Animal Care and Use Committee at Loma Linda University. The online-only Data Supplement contains detailed methods. Stereotoxic infusion of 0.3 U bacterial collagenase into the right ganglionic eminence was performed to induce GMH in P7 rat pups, as described. Although some consider P7 rat pups to be equivalent in brain development of a term human infant, recent evidence suggests P7 is closer in brain development to 30 to 32 week gestation age human infants, which is approximately the point we want to model GMH. Forty animals were divided into 4 groups: Sham, Vehicle (phosphate-buffered saline intraperitoneally BID starting 1 hour post-GMH for 7 days), acute deferoxamine (100 mg/kg intraperitoneally BID starting 1 hour post-GMH for 7 days), and delayed deferoxamine (100 mg/kg intraperitoneally BID starting 72 hours post-GMH for 7 days). Each animal group was alternated when undergoing surgeries. Neurocognitive deficits and motor coordination were evaluated by Morris water maze, rotarod, and foot fault tests between 21 and 28 days.
post-GMH (n=10/group). Animals that failed to swim or only swam in stationary circles were excluded from further analysis in our study. Ventricular volume, cortical thickness, white matter loss, and basal ganglia loss were calculated in Nissl stained histological brain sections 28 days post-GMH using National Institutes of Health ImageJ software (n=5/group). Cerebral structure borders were delineated using previously defined criteria from stereologic neuroanatomical studies using optical dissector principles. \(^{9,10}\) Volumes were calculated as the average delineated area from 10 \(\mu\)m sections taken at \(\approx 2.5\) mm, 1.2 mm, 0.7 mm rostral, and 2.9 mm caudal of bregma multiplied by the depth of the cerebroventricular system. Fibronectin and vitronectin expressions were quantified by Western blot at 28 days post-GMH (n=5/group). Blinded investigators performed the neurobehavioral, histological, and Western blot analyses.

Sample size estimations were made by power analysis using a type 1 error rate of 0.05 and a power of 0.8 on a 2-sided test. Data are expressed as mean±SEM. Western blot and histological data were analyzed by 1-way ANOVA followed by the Tukey post hoc test, expressed as mean±SEM. Western blot analyses were conducted at 28 days post-GMH 

### Results

#### Deferoxamine Improved Brain Morphology After GMH

At 28 days post-GMH, brain morphology was assessed in Nissl stained brain sections (n=5 group). Ventricular volume was found significantly increased in the vehicle group compared with sham, but acute and delayed deferoxamine treatment significantly reduced GMH-induced ventricular dilation (P<0.05; Figure 1A). Cortical thickness was evaluated as a right hemisphere/left hemisphere ratio to the mean of sham and was significantly decreased in the vehicle group compared with sham-operated animals, but acute and delayed deferoxamine treatment ameliorated cortical loss (P<0.05; Figure 1B). Basal ganglia volume was evaluated as a percentage to mean sham basal ganglia volume (Figure 1C). Acute and delayed deferoxamine treated groups had significantly less basal ganglia loss compared with the vehicle group (P<0.05; Figure 1C). White matter loss was evaluated as a percent loss using sham-operated animals as a baseline (Figure 1D). Acute and delayed deferoxamine treated groups had significantly less white matter loss compared with the vehicle group (P<0.05; Figure 1D).

#### Deferoxamine Reduced Extracellular Matrix Protein Expression After GMH

Western blot analyses were conducted at 28 days post-GMH induction (n=5 per group). Fibronectin expression was found increased in the vehicle group compared with sham-operated animals, and acute and delayed deferoxamine treatment significantly reduced fibronectin expression after GMH (P<0.05; Figure 2A). Vitronectin expression was also significantly increased in the vehicle group compared with sham-operated animals, and acute and delayed deferoxamine treatment tended to reduce vitronectin expression after GMH (P<0.05 versus sham; Figure 2B).

#### Deferoxamine Improved Long-Term Neurofunctional Outcomes After GMH

Vehicle-treated GMH animals demonstrated significant spatial memory loss compared with sham-operated animals in the Morris water maze by swimming greater distances finding the platform (P<0.05; Figure 3A) and spending less time in the target quadrant during the probe trials (P<0.05; Figure 3B). Acute and delayed treatment animals showed significant cognitive functional recovery by having reduced swimming distances (P<0.05; Figure 3A) and tended to spend more time in the target quadrant during the probe trials (P>0.05 versus sham; Figure 3B). Furthermore, vehicle animals had significantly more foot faults than sham, but acute and delayed deferoxamine treated GMH animals had significantly reduced foot faults compared with vehicle (P<0.05; Figure 3C). Vehicle animals had significantly worse rotarod performances compared with sham, but acute and delayed deferoxamine treated animals had significantly better rotarod performances than vehicle rats (P<0.05; Figure 3D).

### Discussion

Iron toxicity is known to play a crucial pathophysiological role in brain injury after hemorrhage, with some evidence indicating that iron overload may contribute to hydrocephalus development. \(^{3,4,11}\) Blood products and extracellular matrix protein proliferation are thought to contribute significantly to post-hemorrhagic hydrocephalus development by disrupting normal cerebrospinal fluid flow in the ventricles. \(^7\) Deferoxamine treatment attenuated brain injury in adult brain hemorrhage models, yet it has not been evaluated in neonates nor has
it been shown to attenuate neonatal posthemorrhagic hydrocephalus development and long-term neurofunctional deficits significantly. This study evaluated the efficacy of 1 hour (acute) and 72 hour (delayed) deferoxamine treatment as a potential therapeutic modality for GMH-induced brain injury, the delayed time point serving as a more clinically relevant treatment regimen. Extracellular matrix protein proliferation is indicative of gliosis and is hypothesized to deposit within the ventricles, disrupting cerebrospinal fluid flow and contributing to posthemorrhagic hydrocephalus development. Fibronectin and vitronectin were significantly increased in GMH animals compared with sham-operated animals, yet acute and delayed deferoxamine treated animals had significantly reduced fibronectin and vitronectin expressions compared with vehicle, indicating that GMH leads to increased extracellular matrix protein proliferation, which was attenuated by iron chelation.

Additionally, vehicle-treated GMH animals demonstrated significantly enlarged ventricular volume, decreased cortical thickness, increased basal ganglia loss, and increased white matter loss, while acute and delayed deferoxamine treated animals showed significantly improved brain morphological outcomes across all measures. Consequently, acute and delayed deferoxamine treated GMH animals had significantly improved

Figure 2. Western blot analysis of (A) fibronectin and (B) vitronectin at 28 days after germinal matrix hemorrhage. Representative microphotographs of Nissl-stained brain sections (C) at 28 days after germinal matrix hemorrhage (GMH). Values are expressed as mean±SEM. *P<0.05 compared with sham, and #P<0.05 compared with vehicle. N=5 per group. DFX indicates deferoxamine.
Our results corroborate with similar brain hemorrhage animal models showing that deferoxamine treatment ameliorates posthemorrhage brain injury. Furthermore, acute and delayed post-GMH iron chelation significantly reduced neonatal posthemorrhagic ventricular dilation.

Our results suggest that iron toxicity at acute and delayed time points after GMH is associated with posthemorrhagic ventricular dilation, but the pathophysiological mechanisms remain to be elucidated. The choroid plexus is an epithelial layer in the ventricles specialized for cerebrospinal fluid production and is susceptible to injury after hemorrhage. It also contains high expression levels of iron metabolic proteins relative to other brain tissues. Iron overload in the cerebroventricular system after GMH may adversely affect normal functioning of the choroid plexus, leading to pathologically increased cerebrospinal fluid production. Another possible explanation is that GMH-induced cerebroventricular iron overload destroys subarachnoid granulations as is observed in subarachnoid hemorrhage, reducing overall cerebrospinal fluid reabsorption. Further investigations are needed to determine mechanisms linking GMH-induced iron toxicity with long-term posthemorrhagic ventricular dilation.

Our study is the first to show that deferoxamine attenuates long-term neurocognitive and sensorimotor deficits, improves overall brain morphology, and reduces posthemorrhagic ventricular dilation after GMH when treatment is initiated as late as 72 hours postictus in neonates, providing evidence for a potentially clinically translatable therapeutic modality.

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Disclosures
None.

References
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Animal Surgery

Timed-pregnant rats were purchased from Harlan Laboratories (Indianapolis, IN) and experimental germinal matrix hemorrhage (GMH) was induced in P7 rat pups as previously described. Briefly, rat pups were anesthetized with isoflurane (3 % in a 30/70 oxygen/medical air mixture) and placed prone with their head secured onto a rodent stereotaxic frame. After sterilizing the rodent’s scalp, a small midline incision was made to expose bregma. Next, a cranial burr hole was made 1.8 mm rostral and 1.5 mm right lateral from bregma, using a standard dental drill (1 mm). A 26-gauge needle was inserted through the cranial burr hole and stereotactically lowered 2.8 mm into the brain parenchyma. Following that, bacterial collagenase VII-S (0.3 U, Sigma; St. Louis, MO) was infused into the right hemispheric ganglionic eminence, at a rate of 0.25 µl/minute. Back-leakage of collagenase was prevented by keeping the needle in place for 10 minutes after completed infusion. Following that, the needle was withdrawn at a rate of 1 mm/minute, the burr hole was sealed with bone wax, and the scalp sutured. Sham animals were subjected to needle insertion only. Rat pups were returned to their dams after full recovery from the anesthesia.

Behavioral Testing

Neurocognitive deficits and motor coordination were evaluated by Morris water maze, rotarod, and foot fault tests between 21-28 days post-GMH (n=10/group). The mentioned neurofunctional tests have been previously utilized for the evaluation of long-term deficits in rodents subjected to unilateral hemorrhagic brain injury. All tests were conducted in a blinded fashion. Learning and memory abilities in rats were assessed via the Morris water maze by measuring (1) the swim
distance for each animal before detecting a slightly submerged platform in a pool of water (diameter: 110 cm) and (2) the time each animal spent searching the target quadrant, after the platform has been removed from the pool (probe trial). An overhead infrared camera linked to a computerized tracking system (Noldus Ethovision, Tacoma, WA) recorded the swim path and time of each animal. The water maze experiments were conducted on day 21 to 25 post-GMH induction, one block per day followed by the probe trial. Motor and coordination function were evaluated on post-operative days 26 to 28. The rotarod apparatus (Columbus Instruments, Columbus, OH), consisting of horizontally rotating cylinders (7 cm in diameter, 9.5 cm in width) rotate either at constant velocity or accelerate 2 RPM every 5 seconds starting at a speed of 5 or 10 RPM. Continuous walking was required to avoid falling; the latency to fall was recorded for each animal by a photobeam circuit. Foot-fault testing was conducted by placing each animal on a horizontally elevated wire-grid (20 cm x 100 cm) and missteps through the grid were recorded over 2 minutes.

**Histopathological analysis**

Ventricular volume, cortical thickness, white matter loss, and basal ganglia loss were calculated in Nissl stained histological brain sections 28 days post-GMH using NIH Image J software (n=5/group). Brain tissue preparation, staining, and volumetric evaluations were conducted as previously described.  
Briefly, animals under deep isoflurane anesthesia were euthanized by transcardiac perfusion with PBS and 4% paraformaldehyde. Following that, brains were collected, formalin fixed (in 4% paraformaldehyde for 3 days), dehydrated (in 30 % sucrose for 3 days), and frozen coronal brain slices (10 µl) were obtained, using a cryostat (CM3050S; Leica Microsystems). Nissl stained histological brain sections were evaluated via computer assisted (Image J) hand delineation of cerebral and cerebroventricular structures, based on criteria from
stereologic studies using optical dissector principles. The ventricular volume (mm³) was calculated as average ventricular area multiplied by the depth of the cerebroventricular system. The cortical thickness was calculated and expressed as ratio of the contralateral brain cortex. Basal ganglia volumes and white matter loss were expressed as % of the sham group. Basal ganglia and white matter volumes were calculated and expressed as % of the sham group by dividing their volumes to the overall average sham volume.

**Western blotting**

Fibronectin and vitronectin expressions were quantified by Western blot at 28 days post-GMH (n=5/group). Briefly, whole brain samples were processed according to previously published protocols, and equal amounts of protein (50 µg) were separated by SDS-PAGE before being transferred onto nitrocellulose membranes. The latter were then blocked and incubated with the following primary antibodies: anti-fibronectin, anti-vitronectin (1:1000; Abcam) and anti-β-actin (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA). After incubation with the primary and the appropriate secondary antibodies (1:4000; Santa Cruz Biotechnology, Santa Cruz, CA), immunoblots were visualized with the ECL Plus chemiluminescence reagent kit (Amersham Bioscience, Arlington Heights, IL) and bands were quantified using Image J (NIH). Results, expressed as mean±SEM, were normalized to the average values of the sham group.
**Supplemental References**


