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Role of Hemoglobin and Iron in Hydrocephalus After Neonatal Intraventricular Hemorrhage

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Abstract

Background—Neonatal germinal matrix hemorrhage/intraventricular hemorrhage (GMH/IVH) is common and often results in hydrocephalus. The pathogenesis of post-hemorrhagic hydrocephalus is not fully understood.

Objective—To explore the potential role of hemoglobin and iron released after hemorrhage.

Methods—Artificial cerebrospinal fluid (aCSF), hemoglobin, or iron was injected into the right lateral ventricle of postnatal day-7 Sprague Dawley rats. Ventricle size, heme oxygenase-1 (HO-1) expression, and presence of iron were evaluated 24 and 72 hours after injection. A subset of animals was treated with an iron chelator (deferoxamine) or vehicle for 24 hours after hemoglobin injection, and ventricle size and cell death were evaluated.

Results—Intraventricular injection of hemoglobin and iron resulted in ventricular enlargement at 24 hours compared to injection of aCSF. Protoporphyrin IX, the iron-deficient immediate heme precursor, did not result in ventricular enlargement after injection into the ventricle. HO-1, the enzyme that releases iron from heme, was increased in the hippocampus and cortex of hemoglobin-injected animals at 24 hours compared to aCSF-injected controls. Treatment with an iron chelator, deferoxamine, decreased hemoglobin-induced ventricular enlargement and cell death.

Conclusion—Intraventricular injection of hemoglobin and iron can induce hydrocephalus. Treatment with an iron chelator reduced hemoglobin-induced ventricular enlargement. This has implications for pathogenesis and treatment of post-hemorrhagic hydrocephalus.

Keywords

Hemoglobin; hydrocephalus; iron; neonatal

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Introduction

Neonatal germinal matrix hemorrhage/intraventricular hemorrhage (GMH-IVH) remains a significant source of neonatal stroke and death.¹ In addition to brain injury, GMH-IVH results in post-hemorrhagic ventricular dilation and hydrocephalus in up to 25% of very low birth weight infants.² Current therapeutic strategies focus on removing cerebrospinal fluid (CSF) from the ventricles but do not prevent or cure hydrocephalus.³ Furthermore, the mechanisms underlying ventricular enlargement after IVH remain unknown.⁴

It is well-known that intracerebral, subarachnoid, and intraventricular hemorrhage all result in injury to the brain, in part through lysis of red blood cells releasing hemoglobin and ultimately iron.^{1, 5-7} In adult rodent models, hemoglobin degradation products result in brain edema after intracerebral hemorrhage (ICH)^{2, 8} and iron plays a role in brain injury after IVH.^{3, 5} Previously reported neonatal animal models of GMH-IVH examined the response to intraventricular blood either by direct injection into the ventricle^{4, 9} or by spontaneous induction in premature animals¹⁰; however, specific components of blood have not yet been evaluated in such a model. After IVH in a rabbit pup model, hemoglobin metabolites were present in the CSF and associated with increased levels of inflammatory markers.¹¹ In another study, iron was present in the CSF of preterm infants with post-hemorrhagic ventricular dilation.¹² However, the role of specific components of blood in hydrocephalus after IVH in neonates has not yet been explored.

Given that clinical rates of ventricular dilatation and the need for surgical treatment of hydrocephalus are related to the presence of IVH, and that hemoglobin and iron are prominent components of blood found in the CSF after IVH, we sought to determine if hemoglobin and iron in isolation could produce ventricular enlargement. By examining specific components of blood within the ventricular system, we aimed to elucidate the mechanism by which IVH results in hydrocephalus.

Methods

Animals and Intraventricular Injection

Animal use protocols were approved by our institution's Committee on the Use and Care of Animals. Postnatal day-7 (P7) Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) were used for all intraventricular injections. Brain development, including myelination and neurogenesis, of a 7-day-old rat is similar to that of a 35-week gestation neonate.^{13, 14} The germinal matrix (which is thought to be the source of hemorrhage in neonatal hemorrhagic stroke) is still present in rats of this age, and begins to involute after this time. Rats were kept in a 12-hour light/12-hour dark environment and given ad libitum access to water (mother rats) or maternal milk.

P7 rats were anesthetized with either isoflurane (3.0% induction, 1.5% maintenance) or pentobarbital (40-50 mg/kg) and placed in a stereotactic head-holder (Kopf Instruments, Tujunga, CA) on a cotton surface with a heated pad. The ear bars were placed and tightened at the external auditory meatus bilaterally. A 5-mm midline incision was made exposing the skull, and bregma was identified using the visualized superior sagittal sinus (midline) and

palpation of the coronal suture with fine forceps as intersecting landmarks. Stereotactic coordinates from bregma (1.7 mm lateral, 0.5 mm anterior, 2.0 mm deep) were used for the injection site into the right lateral ventricle. Injections were performed using a 30-gauge needle (or 26-gauge needle for 4 mM iron [Fe(III)Cl] injections) and a 1-mL syringe attached to a microinfusion pump (World Precision Instruments, Sarasota, FL) at a rate of 8 μ L/minute. The needle was left in place for several minutes after the injection prior to being removed to prevent egress of the injected substance. The incision was closed with 5-0 nylon suture. Animals were returned to the cage with their mother and allowed to recover. There was no difference in ventricular volumes for each condition between the 2 anesthetics. Six of 29 animals who received pentobarbital anesthesia died; 4 died at time of surgery, and 2 died by day 1. There were no deaths in the animals who received isoflurane anesthesia.

All intraventricular injection volumes were 20 μ L. Injections in animals used for experiments included artificial CSF (aCSF; n = 31), bovine hemoglobin (MP Biomedicals, Santa Ana, CA; 50 mg/mL [n=10]; 100 mg/mL [n=4]; 150 mg/mL [n = 16]), Fe(III)Cl (Sigma-Aldrich, St. Louis, MO; 0.5 mM [n = 5], 2 mM [n = 3], 4 mM [n = 17]), or protoporphyrin IX (Sigma-Aldrich; 4 mM [n = 4]). Animals were excluded from analysis if the injection was not within the ventricle. Injections in excluded animals included aCSF (n = 1), 150 mg/mL bovine hemoglobin (n = 1), 0.5 mM Fe(III)Cl (n = 1), and 4 mM Fe(III)Cl (n = 3). Ten animals received anesthesia only. The doses of hemoglobin, iron, and protoporphyrin IX were based on the concentration of hemoglobin found in blood (~140 mg/mL) and the amount of iron and protoporphyrin in hemoglobin.

aCSF contained: 125.5 mM NaCl, 20 mM NaHCO₃, 2.4 mM KCl, 0.5 mM KH₂PO₄, 1.1 mM CaCl₂•2H₂O, 0.85 mM MgCl₂•6H₂O, 0.5 Na₂SO₄, 5 mM glucose; pH 7.4. Bovine hemoglobin and Fe(III)Cl were dissolved in aCSF.

Deferoxamine Treatment

To examine the potential benefit of iron chelation, the effects of deferoxamine were studied. A separate group of animals was given 100 mg/kg deferoxamine (n = 13) or vehicle (sterile water; n = 13) via intraperitoneal (IP) injection 2 hours following intraventricular injection of 150 mg/mL hemoglobin and then *bis in die* (BID) for 24 hours. The total volume of injection was dependent on rat weight. The concentration of the injected solution was 50 mg/mL and the total amount given was 100 mg/kg, which is 40 μ L for a 20g rat.

MRI and Volume Measurement

At 24 or 72 hours after intraventricular injection, T2-weighted MRI sequences were obtained using a 7.0T Varian MRI scanner (183 mm horizontal bore; Varian, Inc., Palo Alto, CA). Animals were anesthetized with isoflurane (1.5%)/air mixture throughout image acquisition, and body temperature was maintained at 37°C by circulating heated air. T2 fast spin-echo sequences (TR 4000/TE 60 mS, FOV 20×20 mm, matrix 256×128, 25 axial slices, 0.5 mm thick) were used to obtain imaging of the entire ventricular system. Image analysis was performed using Image J software (<http://rsbweb.nih.gov/ij/index.html>). T2 images of the brain from anterior to the frontal horns of the lateral ventricles through the 4th ventricle were used for volume calculation. The ventricle was outlined on each slice and the area was

calculated. All measurements were performed by an observer blinded to the treatment group. Ventricular volumes were calculated by adding the ventricle area present on each slice multiplied by slice thickness (0.5 mm).

Western Blot Analysis

Animals were anesthetized with supratherapeutic pentobarbital and perfused with 0.1 M phosphate-buffered saline (PBS), pH 7.4. Brains were removed, and the cerebellum, right and left hippocampi, basal ganglia, olfactory bulbs, and periventricular cortical regions were dissected and flash-frozen in liquid nitrogen. Western sample buffer was added to brain tissue samples, protein concentration determined, and Western blot analysis performed as previously described.¹⁵ Briefly, 50 µg of protein was denatured by heating to 100°C for 5 minutes and then loaded into columns of a 4% stacking/12% poly-acrylamide gel and separated. Gels were transferred to a nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ) overnight at 4°C. Membranes were then blocked in 5% Carnation nonfat dry milk in tris-buffered saline with Tween 20 (TBST), pH 7.6, for 1 hour at room temperature, washed 3 times with TBST, incubated with 1:2000 polyclonal rabbit anti-rat HO-1 antibody (Enzo Life Sciences, Farmingdale, NY) in 2.5% bovine serum albumin for 1 hour at room temperature, washed 3 times with TBST. The secondary antibody (anti-rabbit) was diluted 1:1000 in 2.5% bovine serum albumin (BSA) in TBST buffer and the blot incubated for 1 hour. Membranes were then washed 3 times with TBST buffer, developed using Lumi-Light Western Blotting Substrate (Roche, Nutley, NJ) and visualized in a FluorChem M imager (Protein Simple, Santa Clara, CA). Protein band signals were analyzed using Image J software (<http://rsbweb.nih.gov/ij/index.html>).

Immunohistochemistry/Perls' Staining

Animals were anesthetized with supra-therapeutic pentobarbital and perfused with 4% paraformaldehyde in 0.1 M PBS, pH 7.4, and then decapitated and brains removed. Brains were incubated in the same solution for 24-48 hours at 4°C, then transferred to a solution of 30% sucrose in 0.1 M PBS and incubated at 4°C until brains sank to the bottom. Brains were then embedded in optimal cutting temperature compound (Sakura Finetek USA, Inc., Torrance, CA), frozen at -80°C, then 18-µm thick frozen sections were cut using a cryostat.

For immunohistochemistry, slides were dried with a hair dryer and incubated at room temperature in 0.01M PBS, pH 7.4, with 0.3% Triton 100 (Sigma-Aldrich) for 15 minutes, washed 2 times with PBS, then incubated at room temperature with 1:10 goat serum in PBS. Slides were incubated overnight at 4°C in 1:400 polyclonal rabbit anti-rat HO-1 antibody (Enzo Life Sciences). Slides were washed in PBS 2 times and incubated in a 2:1 PBS/methanol mixture with 0.3% H₂O₂ for 20 minutes. Slides were washed in PBS 3 times and incubated in 1:1000 goat anti-rabbit antibody for 90 minutes. After washing 3 times in PBS, slides were incubated for 1-3 minutes in 3,3'-diaminobenzidine-4HCl (Liquid DAB Substrate Kit; Invitrogen, Carlsbad, CA), dehydrated, and cover-slipped with Permount mounting medium (Fisher Scientific, Waltham, MA).

Ferric iron was visualized using Perls' stain as previously described.¹⁶ Briefly, slides were dried with a hair dryer for 15 minutes and incubated for 30 minutes in a 1:1 mixture of 4%

potassium ferrocyanide and 1.2 mM HCl. Slides were then rinsed 3 times in deionized water and incubated for 60 minutes in methanol containing 0.01 M NaN_3 and 0.3% H_2O_2 . Next, slides were washed in 0.01 M PBS, pH 7.4, incubated for 30 minutes in 3,3'-diaminobenzidine-4HCl (Liquid DAB Substrate Kit; Invitrogen), washed 3 times in deionized water, then dehydrated and cover-slipped.

Potassium Determination

Animals were anesthetized with supra-therapeutic pentobarbital and decapitated. Brains were removed, separated into the cerebellum and right and left cerebral hemispheres, dried in a gravity oven at 90°C for 48 hours, and weighed on an electronic analytical scale to obtain the dry weight. Samples were digested in 1 mL of 1 N nitric acid for 1 week and potassium content measured with an automatic flame photometer (Instrumentation Laboratory, Bedford, MA).

Statistical Analysis

Data are presented as mean $\pm$ SEM. Two-group comparisons were performed using *t* test, and ANOVA with Dunnett post-hoc test was used for comparison of more than 2 groups. Pearson's correlation was used for iron dose response correlation with ventricular volume. *P* value less than 0.05 was considered significant.

Results

Intraventricular Injection of Hemoglobin

In P7 rats, intraventricular injection of 20 μL hemoglobin (150 mg/mL), but not aCSF, resulted in lateral and 4th ventricle dilation at 24 hours (Figure 1A). The frontal horns of anesthesia control animals (no injection) and aCSF-injected animals appeared hypointense on T2-weighted MRI from choroid plexus filling the frontal horn, which differs from the hyperintense appearance of increased CSF after hemoglobin injection. As there was a marked increase in ventricular volume after injection of hemoglobin, we quantified total ventricular volume 24 hours after ventricular injection of different concentrations of hemoglobin and found that increasing concentrations resulted in a larger ventricle size compared with aCSF (6.2 ± 1.2 vs. $1.7 \text{ mm}^3 \pm 0.1$; $P < 0.0001$; Figure 1B). Animals that received anesthesia only and those injected with aCSF had similar ventricular sizes (1.5 ± 0.1 vs. $1.7 \text{ mm}^3 \pm 0.1$) that were markedly smaller than hemoglobin-injected animals. Ventricle size was not affected by type of anesthesia (See Figure, Supplemental Digital Content).

Intraventricular Injection of Iron or Protoporphyrin IX

Because iron, contained within heme, is a principal component of hemoglobin and may play a role in ventricle dilation and hydrocephalus after IVH, the effects of iron alone were examined. Fe(III)Cl in 3 different concentrations (0.5, 2, and 4 mM) was injected into the right lateral ventricle of P7 rats. At 24 hours after injection, T2-weighted MRI revealed a progressive increase in ventricle volume with increasing iron concentration (Figure 2A-B; $r = 0.80$; $P < 0.0001$). The frontal horns appear dark after iron injection due to the ferromagnetic properties of iron.

In hemoglobin, iron is contained within a heme ring. To examine whether the ring itself might also contribute to hemoglobin-induced hydrocephalus, the effects of protoporphyrin IX, the iron-deficient immediate precursor to heme, was examined. Intraventricular injection of 4 mM protoporphyrin IX did not cause an increase in ventricle volume when compared to aCSF control (1.4 ± 0.2 vs. 1.7 ± 0.1 mm³; $P = 0.08$; Figure 2C).

Brain HO-1 After Hemoglobin Injection

HO-1 is a key enzyme that breaks down heme into iron, carbon monoxide, and biliverdin. We evaluated HO-1 protein levels in the ipsilateral hippocampus and periventricular cortex and found significant increases 24 hours after intraventricular hemoglobin (50 mg/mL) injection compared to either aCSF or Fe(III)Cl (4 mM) injection (Figure 3A-B). HO-1 immunohistochemistry revealed intense periventricular staining at both 24 and 72 hours after hemoglobin injection (150 mg/mL), when compared to aCSF controls (Figure 4A). HO-1 was also present in the corpus callosum and contralateral periventricular area in hemoglobin-injected animals.

Iron within the Subventricular Zone After Intraventricular Hemoglobin Injection

As there were increased brain HO-1 levels after intraventricular hemoglobin injection, the effects on brain iron were also examined. DAB-enhanced Perls' staining demonstrated bilateral periventricular iron 24 and 72 hours after hemoglobin injection. Iron was also present within the subventricular zone, a site of ongoing cell proliferation in the neonate (Figure 4B).

Time Course of Ventricular Enlargement After aCSF, Hemoglobin, or Iron Injection

Injection of either hemoglobin or iron results in ventricular enlargement 24 hours after injection. In addition, iron is present in the parenchyma at both 24 and 72 hours after hemoglobin injection. Therefore, we examined differences in ventricular size over time between the iron- and hemoglobin-injected animals, as evaluated by T2-weighted MRI. Ventricle volume continued to increase in the hemoglobin group from 24 to 72 hours (6.2 ± 1.2 vs. 13.0 ± 2.1 mm³; $P < 0.01$), but decreased in the aCSF group (1.7 ± 0.1 vs. 1.4 ± 0.1 mm³; $P < 0.05$), and remained stable in the iron group (4.3 ± 0.4 vs. 3.6 ± 0.8 mm³; $P = 0.2$) (Figure 5A-B).

Treatment with an Iron Chelator Following Hemoglobin-Induced Ventricular Enlargement

To further evaluate the potential role of iron in hemoglobin-induced ventricular enlargement, the effect of an iron chelator, deferoxamine, was examined. Rats were treated with deferoxamine (100 mg/kg, IP) or vehicle 2 hours after intraventricular injection of hemoglobin (150 mg/mL) with continued treatment BID for 24 hours. Deferoxamine treatment reduced total ventricular volume at 24 hours (2.3 ± 0.3 vs. 3.5 ± 0.5 mm³; $P < 0.05$; Figure 6A-B). Cell death in the ipsilateral and contralateral cerebral hemispheres was also assessed at 24 hours by determining tissue K⁺ levels (a primarily intracellular ion that is lost from the brain after injury). Ipsilateral tissue K⁺ was higher in deferoxamine-treated rats compared to vehicle (614 ± 7 vs. 592 ± 6 μmol/gm dry weight; $P < 0.05$) and similar to the contralateral hemisphere (Figure 6C).

Discussion

IVH is a cause of significant morbidity and mortality in premature neonates,^{1, 17} with 12,000 new cases each year in the United States (www.cdc.gov). Hydrocephalus develops in up to 1/3 of patients with IVH and often requires lifelong treatment.^{2, 18} There are no targeted preventive treatments for hydrocephalus due to our lack of understanding of how IVH results in ventricular enlargement. In this study, we demonstrate that intraventricular hemoglobin and iron, components of red blood cells, are capable of inducing ventricular dilation in neonatal rats and that iron chelation reduces hemoglobin-induced ventricular enlargement. We also demonstrate that iron plays a key role in neonatal IVH-associated hydrocephalus, and propose a therapeutic treatment strategy to reduce acute ventricular enlargement. By understanding the mechanisms of hydrocephalus after IVH, we hope to expand the currently limited treatment options for this common neonatal condition.

The mechanism of post-hemorrhagic hydrocephalus is unknown. Although arachnoid granulations were previously implicated in the pathogenesis of hydrocephalus, studies evaluating arachnoid granulations after hemorrhage are limited¹⁹⁻²² and their role in hydrocephalus development after neonatal IVH is unclear, as arachnoid granulations themselves are difficult to identify in the neonatal population.²³ Additional routes of CSF drainage may be affected, but this remains an area of investigation⁴ and the pathogenesis of post-hemorrhagic hydrocephalus remains elusive. It is possible that TGF- β plays a role in obliterative arachnoiditis and hydrocephalus; however, it remains unclear, as inhibitors of TGF- β have failed to reduce ventricular size after IVH.^{24, 25}

Previous studies from our group have shown that hemorrhage and components of blood within the ventricular system result in brain injury and ventricular dilation in the adult.^{5, 6, 26-28} Neonatal animal models of IVH have primarily examined the impact of blood within the ventricle^{9, 29, 30} but have not addressed the role of specific components of blood in the pathogenesis of hydrocephalus. As iron and hemoglobin metabolites are present in CSF after ICH or IVH,^{11, 12, 31} they may play a role in ventricular enlargement. We found a continued increase in ventricle size from day 1 to day 3 after hemoglobin injection, possibly due to continued release of iron, compared with stable ventricle size at days 1 and 3 after iron injection. Existing animal models of neonatal post-hemorrhagic hydrocephalus have examined the response to blood within the ventricle^{9, 10} using large volumes to induce hydrocephalus. To eliminate ventricular distension from a large volume of blood as a reason for an increase in ventricular volume, small volumes are desirable. Our model evaluates 2 specific components of blood, hemoglobin and iron. The majority of animals in our experimental groups (hemoglobin and iron) had ventricular dilation compared to few animals in our control group (aCSF). The small-volume injection (20 μ L compared to 80 μ L $\times$ 2 in other models) reduces the possibility of distention of the ventricles secondary to a large injection volume.⁹ The fact that a smaller volume of hemoglobin (at a similar concentration to blood³²) is capable of causing hydrocephalus may suggest that exposure to hemoglobin and the iron it contains plays a role in ventricular enlargement. It should be noted that hydrocephalus is a subjective term, as there is no ventricle size cut-off that defines the term. It is often used to describe large ventricles resulting in a clinical change.

Our model describes acute changes to the ventricular system, without assessment of the ventricles chronically.

Hemoglobin degrades into heme, which reacts with heme oxygenases (HOs) resulting in the production of iron, carbon monoxide, and biliverdin. HO plays a crucial role in hemoglobin breakdown. While HO-2 is constitutively expressed in brain, HO-1 is normally absent but is inducible. In adult rats, intracisternal hemoglobin causes marked up-regulation of HO-1, which starts at ~4 hours and peaks at 24 hours.³³ In the current study, we also found marked HO-1 induction at 24 hours after intraventricular hemoglobin injection in the neonatal rat. The products of HO activity are biliverdin (which is rapidly converted to bilirubin), carbon monoxide, and iron. Even in the adult, the full-time courses of the hemoglobin metabolites have not been fully determined, but there is evidence that iron remains in the brain long-term after ICH.³⁴ We found increases in HO-1 expression in the hippocampus and cortex after hemoglobin injection, and the release of iron from heme may play a role in ventricular dilation. In adult animals, IVH and ICH cause marked increases in HO-1 expression,^{5, 35-37} as does intraparenchymal injection of hemoglobin.⁸ Our study shows that a similar response occurs in the neonatal rat. In ICH, there is evidence that HO-1 inhibition or gene deletion can be neuroprotective.³⁸⁻⁴⁰ The effect of such inhibition in neonates merits investigation.

Ventricle size increased after injection of iron, but not after injection of the iron-deficient immediate precursor to heme, protoporphyrin IX. Iron results in the production of reactive oxygen species through its reaction with hydrogen peroxide, and this may contribute to increased ventricular size. The ipsilateral ventricle dilated more than the contralateral ventricle, likely due to a higher local iron concentration at the site of injection. In contrast to iron, protoporphyrin IX did not result in an increase in ventricular size and there was a trend toward a decrease in ventricle size. After injection into the ventricle, local injury and micro hemorrhages may occur from the needle traversing the brain. Decrease in ventricular size may result from protective effects of protoporphyrin IX, which is capable of chelating iron and inhibiting HO-1.³⁸⁻⁴⁰

Deferoxamine has been used in neonatal hypoxia-ischemia rodent models as a possible neuroprotectant.⁴¹⁻⁴³ However, its effects on neonatal hemorrhagic stroke and post-hemorrhagic hydrocephalus are unknown. Deferoxamine crosses the blood-brain barrier and accumulates in the brain 1-2 hours after peripheral administration at doses of 100 mg/kg in an ischemic brain injury model.⁴⁴ Intranasal administration is an additional delivery route that bypasses the blood-brain barrier.⁴⁵ Here we found that acute treatment with deferoxamine not only attenuates hemoglobin-induced ventricular enlargement but also decreases ipsilateral hemisphere cell death (Figure 6). The mechanism for its action is unclear. Treatment with deferoxamine may act through its interactions with iron in the ventricle; however, it may also act within the brain parenchyma. Deferoxamine reduces free iron levels in the CSF after adult ICH⁴⁶ and may act through chelation of ferric iron in a similar manner. However, the ability of deferoxamine to alter hypoxia-inducible factor-1-alpha levels and the inflammatory cascade may also play a role.^{47, 48}

While iron excess is harmful and, as we showed, plays a role in hydrocephalus after IVH, treatment with iron chelation should be studied in more depth to ensure that untoward

neurodevelopmental consequences do not occur from possible overcorrection of iron excess. Iron is needed in oligodendrocytes during myelination⁴⁹ and iron deficiency can result in motor and cognitive deficits that are not correctable with supplementation.⁵⁰ Thus, while the effects of deferoxamine in relation to neonatal and adult IVH animal models are promising, further studies are required to investigate this approach, including studies of deferoxamine on the effects of intraventricular blood injection in neonatal animal models.

Neonatal IVH damages and affects development of neuronal and oligodendrocyte progenitor cells⁵¹ that reside in the subventricular zone (SVZ) located adjacent to the ventricle. The SVZ is an important highly cellular area,⁵² particularly in the developing brain of neonates. Thus, damage to this area affects brain development and recent work suggests that primary cilia defects in neural progenitor cells of the SVZ may play a role in hydrocephalus.⁵³ Iron was present in the surrounding periventricular tissue and SVZ after intraventricular hemoglobin injection in our model and may act to damage or alter the production of progenitor cells of the SVZ.

Our model demonstrates changes to the ventricular system acutely (24 and 72 hours after injection) and thus, changes in ventricular size would be expected to result from an increase in ventricular size rather than brain atrophy. Future studies are needed to examine the long-term effects of intraventricular hemoglobin and iron and whether initial ventricular dilation progresses to permanent hydrocephalus.

Conclusion

Intraventricular injection of hemoglobin or iron in neonatal rats results in early ventricular dilation, which is reduced by treatment with the iron chelator, deferoxamine. This represents a potential therapeutic target for treatment of hydrocephalus after neonatal GMH-IVH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Intraventricular injection of hemoglobin (Hb), but not aCSF, results in ventricular enlargement. (A) Representative T2-weighted MRIs of lateral (frontal and temporal horns) and 4th ventricles in anesthesia control animals and aCSF and hemoglobin-injected (150 mg/mL) animals 24 hours after injection. (B) Quantification of ventricle volume 24 hours after no injection, aCSF injection, or injection of different concentrations of hemoglobin (50, 100, or 150 mg/mL) ($n = 4-30$ per group; $*P < 0.0001$; ANOVA with Dunnett post-hoc test for multiple comparisons with CSF used as reference). Mean $\pm$ SEM.

Figure 2.

Iron, a principle degradation product of hemoglobin, results in ventricular enlargement. (A) Representative T2-weighted and T2* MRIs showing that increasing concentrations of Fe(III)Cl result in corresponding increases in ventricle size 24 hours after intraventricular injection. (B) Quantification of ventricle volume in mm³ for different concentrations of Fe(III)Cl (Pearson correlation coefficient, $r = 0.80$; $P < 0.0001$). (C) Iron-deficient protoporphyrin IX (immediate precursor to heme) does not increase ventricular size 24 hours after injection ($n = 4-30$ per group; $*P < 0.0001$; ANOVA with Dunnett post-hoc test for multiple comparisons with CSF used as reference). Mean $\pm$ SEM.

Figure 3.

Increased expression of key iron handling protein, HO-1, after intraventricular injection of hemoglobin. (A) Western blot demonstrating increased levels of HO-1 in hippocampus and cortex 24 hours after hemoglobin injection ($n = 3$ per group). (B) Quantification of HO-1 levels (fraction of actin control) ($n = 3$ per group; $*P < 0.01$; ANOVA with Dunnett post-hoc test for multiple comparisons with CSF used as reference). Mean $\pm$ SEM.

Figure 4.

Periventricular HO-1 and iron after intraventricular injection of hemoglobin. (A) Ipsilateral frontal horn of lateral ventricle showing HO-1 immunoreactivity (scale bar = 500 μ m) and (B) DAB-enhanced Perls' iron staining of ipsilateral ventricle and periventricular zone (scale bar = 200 μ m) 24 and 72 hours after intraventricular injection of hemoglobin.

Figure 5. Ventricle enlargement at 24 and 72 hours after injection of aCSF, hemoglobin, or Fe(III)Cl. (A) Representative T2-weighted MRIs of lateral ventricles 24 and 72 hours after injection. (B) Quantification of ventricle volume (* $P < 0.05$; ** $P < 0.01$; 1-tailed unpaired t test). Mean $\pm$ SEM.

Figure 6.

Peripheral treatment with the iron chelator, deferoxamine (DFX), reduces hemoglobin (Hb)-induced ventricular enlargement and cell death. (A) Representative T2-weighted MRIs of lateral ventricles, cerebral aqueduct, and 4th ventricle in vehicle-treated or deferoxamine-treated animals 24 hours after intraventricular hemorrhage. (B) Quantification of ventricle size in mm³ (n = 13 per group; **P* < 0.05). (C) Cell death measured by K⁺ levels in ipsi- and contralateral hemispheres in vehicle- and deferoxamine-treated animals (n = 4-5 per group; **P* < 0.05; 1-tailed unpaired *t* test). Mean ± SEM.