Characterization of Acute Brain Injuries and Neurobehavioral Profiles in a Rabbit Model of Germinal Matrix Hemorrhage

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Background and Purpose—Germinal matrix hemorrhage-intraventricular hemorrhage (GMH-IVH) is the most common neurological problem of premature infants and has enormous financial and social impact. Despite this, there is no standardized animal model of IVH depicting acute brain injuries.

Methods—We delivered rabbit-pups prematurely at 29-day gestation by C-section, administered intraperitoneal glycerol to the pups at 3-hour postnatal age to induce IVH, and evaluated the brain for evidence of injuries.

Results—About 80% of glycerol-treated pups developed gross IVH. We found greater neutrophil and microglia infiltration around the ventricles (periventricular zone) in pups with IVH than in controls. We noted more apoptosis and neuronal degeneration in the periventricular zone than in the neocortex in pups with IVH, but not in controls. There was evidence of axonal damage revealed by β-amyloid precursor protein and neurofilament immunolabeling. Neurobehavioral testing showed that pups with IVH were more wobbly with lesser capability to walk on inclination than pups without IVH. There was no evidence of acute systemic toxicity in the glycerol-treated pups. An evaluation of autopsy materials from premature infants revealed similar evidence of apoptosis and cellular infiltration in the periventricular zone in cases with IVH, but not in cases without IVH—suggesting clinical relevance of the model.

Conclusion—The study provides an instructive animal model of IVH with evidence of acute brain injuries that can be used to evaluate strategies in prevention of IVH and acute posthemorrhagic complications. (Stroke. 2008;39:3378-3388.)

Key Words: GM hemorrhage-intraventricular hemorrhage ■ inflammation ■ apoptosis ■ neutrophil ■ microglia ■ neurobehavioral testing ■ axonal damage

Germinal matrix hemorrhage-intraventricular hemorrhage (GMH-IVH) is the most common neurological disorder of newborns and occurs in 20% of premature infants (12 240 per year in the United States) of less than 1500 g birth weight.1,2 These infants develop a number of neurological sequelae, including posthemorrhagic hydrocephalus, cerebral palsy (10%), and mental retardation.3 The pathogenesis of GMH is usually attributed to vascular wall weakness of the GM vasculature and fluctuation of cerebral blood flow in unstable preterm newborns.4,5 Vascular wall weakness is ascribed to a relative paucity of pericytes in the GM vasculature resulting from a rapid angiogenesis accompanied by high levels of vascular endothelial growth factor and low levels of transforming growth factor-β1 in the GM.5,6

As GMH-IVH is not a completely preventable disorder and because clinical treatment of GMH is supportive and not therapeutic, it is necessary to develop therapeutic strategies either to minimize IVH or to protect the premature brain from the adverse effects of hemorrhage. Thus, it is critical to characterize brain injuries and the neurobehavioral deficits in a standardized animal model of IVH so that new treatment and preventive modalities can be tested.

Several animal species, including rabbit, beagle, and rodents, have been used to model GMH-IVH.8 We selected prematurely delivered rabbit pups to study IVH because of certain close similarities to the human condition: (1) the maximum growth of brain occurs perinatally, (2) there is abundant GM, (3) the blood flow to the brain is via internal carotid and vertebral arteries, (4) the maturation of the lung is completed just before term making them capable of survival on premature birth, and (5) they are at risk for spontaneous IVH.9–11 A number of interventions, including administration of hyperosmolar or hypertensive agents, induction of hypoxia, hypercapnia or hypervolemia, and injection of blood into the GM, have been done to produce IVH in these animals.12–14 We chose to administer intraperitoneal glycerol (hyperosmolar agent) for induction of IVH in premature rabbit pups because it neither causes direct injury to the brain...
by needle insertion nor confounds the model with unwanted metabolic changes in the neural cells by hypoxic-ischemia or hypercapnia. Glycerol treatment results in dehydrogenation and an increase in serum osmolarity, which is attended by a decline in intracranial pressure and consequential increase in transmural pressure across the vessel wall causing rupture of the vasculature. Importantly, the development of GMH in this model is an effect of intracranial hypotension and not of the osmotic agent per se, as no hemorrhage has been found when a fall in intracranial pressure is prevented by intracisternal infusion of saline.

Intracerebral hemorrhage in the adult rat is attended by a robust inflammatory response. However, injection of blood into the GM of mouse pups results in minimal inflammatory response. The inflammatory changes, cell death, axonal damage, and consequent neurobehavioral effects have not been studied in a rabbit model of glycerol-induced IVH or other models, including beagle pups or newborn rats. On this basis, we sought to further characterize the rabbit pup model of GMH-IVH by determining the frequency of hemorrhage at a particular dose and postnatal age of glycerol administration. We then asked whether induction of IVH in the brain of premature pups could produce inflammatory changes including cellular infiltration and death, and axonal damage and if so, whether it was attended by neurobehavioral abnormalities. We explored the feasibility of diagnosing IVH by ultrasound in premature pups. In addition, we determined short-term adverse-effects of intraperitoneal glycerol on organ systems. Finally, we evaluated the brain of premature infants (post-mortem) with IVH to assess clinical relevance of the model.

Materials and Methods

Animal Experiment

The animal protocol was approved by the Institutional Animal Care and Use Committee of New York Medical College. Timed pregnant New Zealand rabbits were obtained from Charles River Laboratories Inc. (Wilmington, Mass). The pups were delivered by C-section at a gestational age of 29 days (full-term = 32 days). Pups were dried and kept warm in an incubator which was maintained at a temperature of 35°C. Pups were fed 1 mL KMR (PETAG Inc.) at 4 hours of age and then 2 mL every 12 hours (100 to 120 mL/kg/d) using a 3.5 French feeding tube. At the age of 3 hours, the pups alternatively received an intraperitoneal injection of 50% glycerol (6.5 gm/kg) or normal saline (control). Head ultrasound (Acuson Sequoia C256 system, Siemens) was performed 24 hours later to assess for the presence and severity of IVH. We euthanized pups at 24, 48, and 72 hours postnatal age (n=4 at each time point). Brains were sectioned into 2-mm slices starting from the cranial end of the brain and were examined for gross IVH. Brains without gross hemorrhage were evaluated for microscopic hemorrhages after H&E staining of the sections. IVH was classified based on a modification of Papile grading: (a) mild, no gross hemorrhage and hemorrhage detected on microscopy of H&E stained brain sections; (b) moderate, gross hemorrhage into lateral ventricles without significant ventricular enlargement; and (c) severe, IVH with significant ventricular enlargement and/or intraparenchymal hemorrhage. The brain samples were fixed in 4% paraformaldehyde in phosphate buffer saline (PBS) for ~18 hours and then were cryoprotected by immersing into 20% sucrose in 0.01 mol/L PBS buffer for 24 hours followed by 30% sucrose for the next 24 hours. Tissues were frozen after embedding them into optimum cutting temperature compound (OCT). Frozen coronal blocks were cut into 20 μm sections using cryostat and saved at −80°C until use.

Immunohistochemistry

The primary antibodies used in experiments included monoclonal mouse antirabbit Neutrophil (Serotec), monoclonal mouse antirabbit CD11b (Serotec), β amyloid precursor protein (Cell signaling technology), monoclonal pan-axonal neurofilament marker SMI-312 (Covance), monoclonal neurofilament 200 (Sigma), and monoclonal neurofilament 68 (Sigma). Briefly, for neutrophil and microglia immunolabeling, sections were fixed in acetone and incubated with the primary antibodies for 1 hour. After washing in PBS, the sections were incubated with secondary antibody and visualized under fluorescent microscope (Carl Zeiss Inc). To evaluate neuronal degeneration, we performed immunohistochemistry using Tuj-1 (neuronal class III β tubulin, Covance) primary antibody followed by Fluoro-Jade B (Chemicon) staining on fixed brain sections according to manufacturer’s instruction.

Fluorescent In Situ Detection of DNA Fragmentation (TUNEL)

Twelve micron tissue sections were air-dried on slides, hydrated in 0.01 mol/L PBS, and permeabilized for 5 minutes in 1:1 ethanol: acetic acid (−20°C). An ApopTag-fluorescein in situ DNA fragmentation detection kit (Chemicon) was used to visualize TUNEL-labeled nuclei. Tissue sections were counterstained with propidium iodide to visualize all the nuclei.

Quantification

We quantified neutrophil and microglia infiltration, apoptosis, and neuronal degeneration in pups with IVH compared to healthy controls. Because brain hemorrhage and attendant cellular infiltration with apoptosis were nonuniform in distribution and were localized to different areas around the ventricle, we chose to count cells in 5 areas—each in the PVZ and cortex—that showed high density of cells. Briefly, from each brain, a set of 3 to 5 coronal sections were taken as every tenth section at each of 2 levels—medial septal nucleus and posterior ventrolateral thalamic nucleus. From every section, about 5 images were acquired from both periventricular zone (PVZ) and cortex using 40× objective. Thus we counted 60 to 100 (5 images×3 to 5 sections×2 regions×2 coronal levels) images per brain. PVZ at the level of medial septal nucleus included GM, caudate nucleus, corona radiata, and corpus callosum; and PVZ at the level of posterior ventrolateral nucleus of thalamus comprised hippocampus, fimbria, GM, internal capsule, thalamus and corona radiata. We evaluated 4 rabbit pups at each time point (24, 48, and 72 hour) in both groups, pups with and without IVH (control). The cell profiles were counted under a fluorescent microscope equipped with a counting grid using a 40× objective.

Caspase 3/7 Activity

We used the Caspase-Glo 3/7 Assay kit (Promega) to measure Caspase 3/7 activity in the tissue. Briefly, forebrain extract were treated with luminogenic caspase-3/7 substrate and luminometer reading was taken at 1 hour. We compared caspase-3/7 activity between the forebrain of pups with IVH and controls.

Triphenyl Tetrazolium Chloride (TTC) Staining

The brain slices of 1 to 2 mm thickness were stained with 2% TTC to assess for necrosis. Specifically, the slices were immersed in a 2% solution of TTC at 37° (pH 7.4) for 15 minutes. A lack of TTC staining was considered to be a sign of necrosis.

Western Blot Analyses

For Western blot analysis, we used a 1-mm-thick slice through the forebrain at the level of the midseptal nucleus and the head of caudate (n=4 pups). Protein extraction and Western blotting were performed under reducing conditions as described before. Briefly, frozen brain tissue was homogenized in sample buffer and boiled for 5 minutes. The protein concentration was determined. Total protein was separated by SDS-PAGE. The separated proteins were transferred to polyvinylidene difluoride membrane by electro-transfer.
The membranes were then incubated with primary antibodies, washed, incubated with secondary antibody, and detected with chemiluminescence ECL system (Amersham).

**Neuro-Behavioral Examination**

We performed neuro-behavioral testing on postnatal day 1 and 3 based on a modification of neurobehavioral scoring protocol by Derrick et al. The testing was performed by 2 blinded physicians. The pups were evaluated inside the infant incubator except for locomotion at 30° angle. Neurobehavioral test was selected based on the capabilities of the premature pups at 24 and 72 hours of age. We evaluated cranial nerves by testing smell (aversive response to alcohol), sucking & swallowing (formula given with a plastic pipette), and head turn to feeding. The responses were graded on a scale of 0 to 3, 0 being the worst response and 3 the best response. Motor examination included tone (modified Ashworth’s scale), motor activity, locomotion at 30° angle, righting reflex, and gait. Tone was assessed by active flexion and extension of forelegs and hindlegs (score 0 to 3). The righting reflex was evaluated by their ability and rapidity to turn prone when placed in supine position. Gait was examined based on ability to crawl and number of roll-overs to either side during locomotion. Sensory examination was limited to touch (touching the face with cotton swab on both sides) on face and extremities and pain on extremities (mild pinprick). Grading of tone, locomotion at 30° slope, and gait are described in the footnote of the Table.

**Human Subjects**

The Institutional Review Board at New York Medical College, Valhalla, NY approved the use of autopsy materials from premature infants (23 to 27 week gestation) for this study. Only autopsy samples <18-hour postmortem interval were used. All infants with major congenital anomalies, chromosomal defects, culture-proven sepsis, meningitis, hypoxic-ischemic encephalopathy, and infants receiving extracorporeal membrane oxygenator treatment were excluded. We included 6 premature infants with grade moderate to severe IVH (5 infants grade 3 and 1 infant grade 4) and 4 premature infants without IVH who died at postnatal age of 1 to 5 days. We used rabbit polyclonal myeloperoxidase (Thermo Scientifics), monoclonal antihuman CD68 (Dako-Cytomation), and rabbit polyclonal caspase 3 (R&D) as primary antibodies.

**Results**

**Intraperitoneal Glycerol and Incidence of IVH**

To determine the incidence and severity of IVH in glycerol-treated rabbit pups, we alternatively assigned 12 litters of pups to receive either intraperitoneal glycerol (6.5g/kg) or saline at 3-hour postnatal age. Pups were killed at 48-hour age and brains were evaluated for IVH. Among glycerol treated pups, 15% pups developed mild (microscopic) IVH, 41% moderate, and 39% severe IVH (Figure 1); only 5% pups did not develop IVH. In contrast, among saline-treated pups, 5% developed moderate and 9% mild IVH; none of the pups had severe IVH in the saline-treated group (supplemental Table I, available online at http://stroke.ahajournals.org). The source of ventricular blood was difficult to discern in pups with severe and moderate IVH, as part of the choroid plexus and GM was typically destroyed. However, in pups with mild IVH (microscopic hemorrhage in GM), we noted congestion and some hemorrhage in the choroid plexus in about one-third of such cases on histology. To determine the feasibility of head ultrasound for identification of IVH, we performed head ultrasound on glycerol treated pups at 8- and 24-hour

### Table. Neurobehavioral Evaluation of Premature Pups at 24- and 72-Hour (E30 and 32) Postnatal Age

<table>
<thead>
<tr>
<th>System</th>
<th>Test</th>
<th>Control D1</th>
<th>IVH D1</th>
<th>P Value D1</th>
<th>Control D3</th>
<th>IVH D3</th>
<th>P Value D3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cranial nerve</td>
<td>Aversive response to alcohol</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
<td>ns</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Sucking and swallowing</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
<td>ns</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Head turns away on touching</td>
<td>3 (2,3)</td>
<td>3 (2,3)</td>
<td>ns</td>
<td>3 (2.5,3)</td>
<td>2.5 (2,3)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>angle of mouth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Motor</td>
<td>Motor activity</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Head</td>
<td>3 (2,3)</td>
<td>3 (2,3)</td>
<td>ns</td>
<td>2.5 (2,3)</td>
<td>3 (2.5,3)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Forelegs</td>
<td>2.5 (2,3)</td>
<td>3 (2,3)</td>
<td>ns</td>
<td>3 (2,3)</td>
<td>3 (2,3)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Hind legs</td>
<td>3 (2,3)</td>
<td>3 (2,3)</td>
<td>ns</td>
<td>3 (2,3)</td>
<td>3 (2,3)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Righting reflex*</td>
<td>4 (3,4)</td>
<td>3 (3,4)</td>
<td>ns</td>
<td>3 (3,4)</td>
<td>3 (3,4)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Locomotion on 30° inclination†</td>
<td>1 (1,2)</td>
<td>1 (1,1)</td>
<td>.001</td>
<td>1 (1,2)</td>
<td>1 (1,1)</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>Tone‡ (Both fore- and hind-limb)</td>
<td>0 (0,0)</td>
<td>0 (0,0)</td>
<td>ns</td>
<td>0 (0,0)</td>
<td>0 (0,0)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>No. of roll-overs to its side during first 10 steps</td>
<td>8.5 (6,9)</td>
<td>9 (7,5,9)</td>
<td>ns</td>
<td>3 (2,5)</td>
<td>8 (7,9)</td>
<td>0.001</td>
</tr>
<tr>
<td>Gait§</td>
<td></td>
<td>1 (1,1)</td>
<td>1 (1,1)</td>
<td>ns</td>
<td>2 (1.5,2)</td>
<td>1 (1,2)</td>
<td>0.001</td>
</tr>
<tr>
<td>Sensory</td>
<td>Facial touch</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
<td>ns</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Pain</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
<td>ns</td>
<td>3 (3,3)</td>
<td>3 (3,3)</td>
<td>ns</td>
</tr>
</tbody>
</table>

Values are median and interquartile range. 0 is the worst response and 3 is the best response.

*Score (range 1–5): No. of times turns prone within 2 seconds when placed in supine out of 5 tries.
†Score (range 0–3): 0, No locomotion; 1, walks, but rolls over on its side with more than 50% of steps; 2, walks, but roll over on its side with less than 50% of steps.
‡Score (range 1–3): 0, No increase in tone; 1, slight increase in tone; 2, considerable increase in tone; 3, Limb rigid in flexion or extension.
§0, No locomotion; 1, walks, but rolls over on its side with more than 50% of steps; 2, walks, but roll over on its side with less then 50% of steps.
ns indicates not significant.

Statistics and Analysis

Data are expressed as mean±SEM. The parameters were compared between pups with and without IVH as well as a function of postnatal age, 24, 48, and 72 hours. We used t test (parametric variable) or Mann–Whitney U test (nonparametric variable) to perform pairwise comparison and ANOVA to compare multiple groups. A probability value of <0.05 was considered significant.
postnatal age using Acuson Sequoia C256 system (Siemens) and subsequently, diagnosis of IVH was confirmed after sacrificing the pups. We diagnosed severe \( n=23 \) and moderate IVH \( n=24 \) with 100% and 92% accuracy respectively compared to autopsy findings. (Figure 1D). However, microscopic (mild) hemorrhages could not be diagnosed with certainty by head-ultrasound. Physiological variables were measured on these pups (supplemental Table I).

**Neutrophil and Microglial Infiltration in IVH**

We next evaluated inflammatory changes in the brain of glycerol-treated pups with moderate to severe IVH compared to saline-treated controls (No gross IVH). To this end, we immunolabeled cryosections from pups with moderate and severe IVH at 24-, 48-, and 72-hour postnatal age using rabbit-neutrophil and -microglia (CD11b) specific antibodies. We observed that immunoreactivities for neutrophils were more around the ventricles (PVZ) than in the cortex. The immunosignals colocalized with nuclear staining by sytox (Figure 2A). Quantification of neutrophil infiltration showed that neutrophil count was higher in pups with IVH than controls in the PVZ at all the 3 epochs—24, 48, and 72 hours \( P<0.001 \) each, Figure 2B). However, neutrophil count in the cortex was not significantly greater in pups with IVH compared to controls. Of note, neutrophil number was greater in the PVZ than in the cortex at all 3 time points, 24, 48, and 72 hours \( P<0.029, 0.001, \) and \( 0.001 \), respectively). In addition, neutrophil count increased significantly with advancing postnatal age, peaking at 72 hours \( P<0.05 \). Likewise, microglia were abundant in the PVZ and scarce in the cortex in pups with IVH (Figure 2C). Our analysis confirmed that microglial infiltration was greater in IVH than controls in the PVZ at 48 and 72 hours \( P<0.01 \) each, but not at 24 hours. Microglia count was significantly higher in the PVZ than in the cortex at 48 and 72 hours \( P<0.012 \) and \( 0.005 \), Figure 2D). In the cortex of pups with IVH, microglial density was not significantly greater than controls. In PVZ of pups with IVH, microglial density increased with advancing postnatal age, peaking at 72 hour \( P<0.05 \). A significant cellular infiltration was not observed in those glycerol-treated pups, who did not develop gross IVH (data not shown).

**Apoptosis and Neuronal Degeneration Abundant Around the Ventricle in IVH**

To assess apoptosis, we performed TUNEL staining on fixed tissue sections and caspase 3/7 activity assay in the brain homogenates of pups with IVH compared to saline-treated controls (No gross IVH). TUNEL staining showed greater density of apoptotic cells in pups with IVH compared to controls in the PVZ \( P<0.001 \) each) and cortex \( P<0.05 \).
each) at all 3 time points (Figure 3A and 3B). In the PVZ of pups with IVH, the largest number of apoptotic cells was noted at 24 hours and decreased significantly with increasing postnatal age in the PVZ \((P < 0.05)\), but not in the cortex. Predictably, number of apoptotic cells was higher in the PVZ than in the cortex at 24- and 48-hour postnatal age \((P < 0.01)\) each, but not at 72-hour. We measured caspase-3 and -7 activity in the brain tissue homogenate of the second 2-mm slice from the rostral end of the forebrain of pups with IVH \((n=11)\) as well as controls \((n=6)\) at 24-hour postnatal age. We found that Caspase-3 and -7 activity was higher in the brain of pups with IVH \((P < 0.02)\) than controls.

We then evaluated brain sections for degenerating neurons. We found that Fluoro-Jade B \((+)\) neurons were more frequent in pups with IVH than controls both in PVZ \((P < 0.001)\) each and cortex \((P < 0.05)\) each at all 3 time points (Figure 3C and 3D). Intriguingly, the degenerating neuronal count decreased with increasing postnatal age in the cortex and PVZ \((P < 0.05, \text{ANOVA})\). Degenerating neurons were more in the PVZ than in the cortex at all 3 time points \((P < 0.01)\) each. In addition, significant apoptosis and neuronal degeneration was not observed in glycerol-treated pups without gross IVH, just as in saline-treated controls (data not shown). Importantly, there was no discernible necrosis on TTC.
staining of brain slices and H&E staining of brain sections (n=4).

**Evidence of Axonal Damage**

To determine axonal damage secondary to GMH-IVH, we evaluated expression of amyloid precursor protein (APP), neurofilament (NF)200 and NF68 by both Western blot analysis (Figure 4A) and immunohistochemistry (Figure 4B) using their specific antibodies. Western blot analysis showed that APP concentration was comparable between glycerol-treated pups with IVH and saline-treated controls (no gross IVH) at 4-, 24-, and 48-hour age. In addition, it did not change significantly with an increase in the postnatal age in both groups of pups. Of note, immunohistochemistry showed APP-positive axonal bulbs and swellings in both superficial and deep white matter at all 3 epochs, 4-, 24-, and 48-hour postnatal age. NF200 levels were similar between glycerol-treated pups with IVH and controls (no IVH) on Western blot analysis. However, it increased with advancing postnatal age in both the control and IVH pups (P=0.024). Immunolabeling of brain sections revealed disrupted and disorganized axonal bundles along with NF200 positive varicosities in the white matter of pups with IVH, whereas axonal bundles appeared smooth, organized, and regular in controls. Western blot analysis for NF68 revealed that NF68 concentration was significantly reduced in pups with IVH than in controls at 8-hour postnatal age, but not at 24- and 48-hour postnatal age. The reason for a transient reduction in NF68 level at 8 hours, but not at subsequent time points, could be neurofilament dephosphorylation rather than protein degradation. In addition, NF68 levels increased with postnatal age in pups with IVH (P=0.002, ANOVA), but not in healthy controls. Like NF200, immunostaining with NF68 depicted disorganized axonal bundles in pups with IVH. We also demonstrated axonal beading and fragmentation in corpus callosum and corona radiata of pups with IVH, using pan-axonal neurofilament marker. No evidence of axonal damage was noted in those glycerol-treated pups who did not develop gross IVH.

![Figure 3. Apoptosis and neuronal degeneration in pups with IVH. A, Representative TUNEL staining of brain sections with IVH. Section counterstained with propidium iodide (PI). Note TUNEL+ nuclei (arrowhead) abundant in the PVZ that colocalized with PI+ nuclei. Lower panel is high magnification image. Scale bar, 100 μm (upper panel) or 20 μm (lower panel). B, Data are mean±SEM n=4 pups at each time point. There was greater density of apoptotic cells in the IVH subjects than controls at all 3 time-points both in the cortex and PVZ. There was more apoptosis in the PVZ than in cortex and 24- and 48-hour, but not at 72-hour age. **P<0.01 cortex vs PVZ of IVH subjects. ###P<0.001 PVZ of IVH subjects vs controls. #P<0.05 cortex for IVH vs controls. C, Representative immunofluorescence of cryosection from rabbit pup brain double-labeled with Tuj1 specific antibody and Fluoro-Jade B. Upper panel depicts Fluoro-Jade B positive (arrowhead) degenerating neurons colocalizing with Tuj-1 positive neurons (arrowhead) in the PVZ of pups with IVH. Note healthy controls do not show neuronal degeneration (lower panel). V indicates ventricle. Scale bar, 20 μm. D, Data are mean±SEM (n=4 pups). Fluor-Jade (+) neurons were more in the pups with IVH than controls in both the PVZ and cortex at each of 3 epochs. Degenerating neurons were more in PVZ compared to cortex at all 3 epochs. Degenerating neurons were greater number at 24 hours than at 48 hours and 72 hours. **P<0.01 cortex vs PVZ of IVH subjects. ###P<0.001 PVZ of IVH vs healthy controls. #P<0.05 cortex of IVH pups vs controls.](http://stroke.ahajournals.org/)
Neurological Signs and Neurobehavioral Testing

Most of the pups looked normal after intraperitoneal glycerol treatment except for some lethargy and poor activity in the pups who developed IVH. About 13% (n=3) of all pups with severe IVH developed seizures, which manifested as retraction of neck and stiffening and jerking of all 4 extremities associated with lack of responsiveness to external stimuli. The duration of these episodes were from <1 minute to

Figure 4. Western blot analysis and immunolabeling for APP, NF200, and NF68 in pups with IVH and healthy controls. A, Western blot analysis showed APP levels were comparable between pups with IVH and healthy controls at 4-, 24-, and 48-hour postnatal age, and it did not change with an increase in the postnatal age significantly in either group of pups. NF200 was also similar between pups with IVH and healthy controls. Note an increase in NF200 with postnatal age in both the healthy and IVH pups. Also note a significant reduction in NF68 levels in pups with IVH than in healthy controls at 4-hour, but not at 24- and 48-hour postnatal age. NF68 levels increased with postnatal age in pups with IVH, but not in healthy controls. *P<0.05 pups with IVH vs controls. B, Immunolabeling with APP specific antibody showed axonal varicosities in the white matter at 24-hour age in pups with IVH, but not in controls. Inset shows high magnification image of axonal varicosity in IVH. Immunostaining with NF200 and NF68 revealed disorganized and sparse axonal-bundles in the internal capsule of pups with IVH, whereas axonal-bundles appeared organized and dense in the internal capsule of control pups. Pan-axonal neurofilament immunolabeling shows axonal beading and fragmentation in corpus callosum and corona radiata of pups with IVH using. Inset depicts high magnification image of axonal beading.

Neurological Signs and Neurobehavioral Testing

Most of the pups looked normal after intraperitoneal glycerol treatment except for some lethargy and poor activity in the pups who developed IVH. About 13% (n=3) of all pups with severe IVH developed seizures, which manifested as retraction of neck and stiffening and jerking of all 4 extremities associated with lack of responsiveness to external stimuli. The duration of these episodes were from <1 minute to
several minutes. We determined the motor and sensory capabilities of 24- and 72-hour-old premature pups delivered at E29 (Table). At 24-hour age, pups both with and without IVH could suck the milk delivered by a plastic pipette and swallow it without nasal regurgitation of the milk. On touching the angle of mouth with the pipette, both groups of pups moved their head away from the stimulus. They were not responsive to light (flashlight) and sound (ringing of bell, clapping) stimuli both at 24- and 72-hour age. All pups typically showed an aversive response to alcohol swabs held close to their nostrils. Motor activity and the ability to turn prone from supine position (righting reflex) was similar in the two groups of pups—with and without IVH—at both 24- and 72-hour postnatal age. To determine the tone of the extremities we performed flexion and extension of the joints and did not observe any difference in tone between pups with and without IVH at both time points. Of note, control pups without IVH at 72-hour age were more active and displayed better ability to walk at 30° inclination compared to pups with IVH. However, this comparison was not significant at 24-hour age. At 24-hour postnatal age, pups with and without IVH were more wobbly and unstable during walking and were noted to tip over to their side with almost every step. However, at 72-hour age, pups without IVH were less wobbly and rolled over to sides less frequently compared to pups with IVH. Those pups that were treated with glycerol and did not develop IVH were similar to pups without IVH on neurobehavioral evaluation (data not shown).

Survival and Systemic Adverse Effects of Intraperitoneal Glycerol

We lost 5 (8.5%) glycerol-treated pups within 72 hours of age. Of these 5 pups, 4 died because of severe IVH manifesting episodes of prolonged seizures and 1 resulting from aspiration of formula during feeding. Among saline-treated controls, 3 pups died <72 hours of age; 1 related to feeding issues and the other 2 were found dead in the morning attributable to unknown reason. Given that i.m. glycerol is attended by rhabdomyolysis and consequent acute renal failure in rats, we evaluated renal function in pups treated with i.p. glycerol. To this end, we measured electrolytes, blood urea, and creatinine in plasma samples of pups treated with glycerol (n=6) and saline (n=6) using Roche Hitachi P-800 autoanalyzer (supplemental Table I). We observed that creatinine levels at 24- and 72-hour postnatal age in glycerol-treated pups were comparable to those of saline-treated controls. However, blood urea was marginally greater in glycerol-treated pups (31.2±3.6 and 33.7±9.6 at 24 hours and 72 hours, respectively) than saline-treated controls (14.5±1.5 and 28.0±2.7 at 24 hours and 72 hours), reflecting dehydration in glycerol-treated pups. Moreover, plasma electrolytes and urinalysis in the glycerol-treated controls were unremarkable, just like saline-treated controls. We have described above that we did not observe significant cellular infiltration, axonal damage, apoptosis, or neuronal degeneration in glycerol-treated pups without IVH, similar to saline-treated controls (no IVH). Likewise, we evaluated intestine, heart, lung, and kidney by gross inspection and microscopy of H&E stained section. These organs did not reveal foci of hemorrhage, necrosis, or any apparent abnormality. However, vessels were congested in these organs at 24-hour but not at 72-hour age.

Clinical Relevance of the Model

To determine whether premature newborns with IVH develop similar inflammatory response secondary to IVH as those of rabbit pups, we evaluated postmortem human materials for apoptosis, neutrophil, and microglia infiltration. TUNEL staining revealed extensive apoptosis in the GM and periventricular white matter of premature infants with IVH, but not in infants without IVH (Figure 5A). Likewise, there was greater expression of caspase-3 in the GM of infants with IVH than in infants without IVH (Figure 5B). Accordingly, immunolabeling of brain sections with myeloperoxidase and CD68 specific antibody showed neutrophil and microglia infiltration in the PVZ of premature infants with IVH, but not in infants without IVH (Figure 5C and 5D). We did not observe cellular infiltration or a significant increase in apoptosis in the cortex of these infants (unlike rabbits). This could be attributable to a proportionately larger area of white matter in humans than in rabbits that may somehow insulate the cortex from the adverse affects of ventricular hemorrhage.

Discussion

As both the survival of premature infants and preterm birth rate have increased significantly, GMH-IVH has developed into a major public concern. However, therapeutic strategies neither in prevention of IVH nor in treatment of posthemorrhagic complications have been adequately explored because of a lack of an appropriate animal model. Herein, we characterize a rabbit pup model of glycerol-induced IVH showing histological evidence of inflammatory changes and cell death. These pups with IVH did not exhibit major neurological deficit (except for wobbly gait and decreased activity) on neurobehavioral testing on days 1 and 3, similar to premature newborns with IVH. Glycerol treatment was not attended by any major cerebral, renal, pulmonary, or other systemic toxicity, which is important for the feasibility of long-term follow-up studies on these animals. Importantly, evaluation of postmortem brain samples from premature infants showed similar inflammatory changes to those of rabbit pups, which suggests clinical relevance of this animal model.

A number of models of perinatal hypoxia-ischemia are available, which have been extensively evaluated. However, GMH-IVH in prematurely delivered animals causing acute brain injuries has not been studied. Our animal model has functional relevance to human conditions and has a number of merits. First, IVH in this model is morphologically similar to IVH in premature infants because hemorrhage usually starts with rupture of blood vessels in the GM vasculature of premature pups and subsequently, ependyma is broken to fill the ventricle with blood. Second, development of IVH in this model is mechanistically similar to those of IVH in preterm neonates because intracranial hypotension and resulting escalation of transmural pressure induced by i.p. glycerol in this model corresponds with the rapid fall in intracranial pressure (fontanel pressure) during first days of life in
Figure 5. Apoptosis and cellular infiltration in premature infants with IVH, just like rabbit pups. A, TUNEL staining showing apoptosis (arrowhead) in PVZ of brain of the infant (25-week gestation) with IVH, but not in infant without IVH (24-week). Scale bar, (upper panel) 100 μm or (lower panel) 20 μm. For B through D, first and second columns are low-magnification images, whereas third and fourth columns are high-magnification images. B, Caspase-3 immuno-staining shows abundant caspase-3 positive signals (arrowhead) in the PVZ of premature infant (25 week) with IVH (2nd column low and 3rd column high magnification), but not in infant without IVH (24-week). Caspase-3 immuno-reactivies (3rd column) colocalizes with sytox (green, fourth column). Inset shows caspase-3 immunolabeling under high magnification. C, Myeloperoxidase (MPO) labeling shows neutrophil (arrowhead) infiltration in PVZ of infants with IVH (2nd column low and 3rd column high magnification), but not in infants without IVH. Signal colocalizes with sytox. D, CD68 labeling depicts microglia (arrowhead) infiltration in PVZ of infants with IVH, but not in infants without IVH. For B–D, Scale bar: left 2 images 50 μm; right 2 images, 20 μm.
premature infants.15,21 Of note, clinical situations, such as a pneumothorax, use of high ventilator pressure (increase in cerebral venous pressure), intravenous bicarbonate (hyperosmolarity), and i.v. furosemide (fall in intracranial pressure) administration, also lead to IVH in preterm newborns secondary to an elevation of transmural pressure across the blood vessel wall. Third, induction of IVH does not require intracranial intervention, such as injecting blood or collagenase, that can potentially traumatize the brain parenchyma despite ultrasound or MRI monitoring.14 Fourth, induction of IVH in the present model results in an inflammatory brain response associated with evidence of cell death and without immediate major neurological deficits, similarly to human premature infants. Fifth, there are a number of advantages of using rabbit as an experimental animal that has been described in the introduction of the paper.9,10 Sixth, this model is relatively less expansive compared to beagles. Collectively, the glycerol-induced hemorrhage in the model resembles IVH of premature infants both mechanically and morphologically, which makes it an attractive tool to study the disease process and evaluation of therapeutic strategies.

This model has a few shortcomings as well. The model involves premature delivery of pups by C-section and subsequently the dam is euthanized. Thus, the orphan pups are nursed in an incubator at a temperature of 35°C and are hand fed using feeding tubes, which is an immense amount of work for the investigators. In addition, gavage feeding the rabbit pups requires a lot of experience and expertise. Furthermore, the rabbit pups are fragile and, unlike rodents, die even with a minor insult or slackness in care. To circumvent these problems, we tried to induce premature labor in the dam using oxytocin so that dam can be saved to rear the pups. Unfortunately, pups could not be consistently delivered vaginally at day 29 because of inadequate and unpredictable response of intramuscular oxytocin, and this approach was abandoned. Another limitation of the model is an associated subarachnoid hemorrhage in more than three quarters of the pups compared to about one quarter of all premature infants with IVH-GMH who show a concurrent subarachnoid hemorrhage.22

The models of IVH in beagle pups have been reported, where IVH was induced either by hemorrhagic hypotension followed by volume reexpansion or by rapidly inducing hypertension with intravenous phenylephrine.12,13 The induction of hemorrhagic hypotension followed by reperfusion leads to the development of IVH in ∼75% of beagle pups and is associated with alteration in cerebral metabolism. The major limitation of this model is the huge cost of the animal and the associated ischemia-reperfusion injury of neural cells confounding the evaluation of brain injuries after IVH. The incidence of IVH in the other beagle pup model is only 40%, in which phenylephrine was used as inducing agent. Another model of IVH in newborn mice used injection of autologous blood into the periventricular tissue of the brain.14 This model had disadvantages of brain damage caused by needle insertion in the brain and chances of missing the periventricular area of the brain during the procedure. Compared to these models, our rabbit model is promising because of use of premature pups, easy induction of IVH, less cost, and relatively few adverse effects after intraperitoneal glycerol treatment.

Four key observations made in the present study were the cellular infiltration, evidence of apoptosis, neuronal degeneration, and axonal damage in the brain of pups with IVH. The finding of greater neutrophil and microglia infiltration at 72 hours compared to 24 and 48 hours is in agreement with the observation in adult rat model of brain hemorrhage.8,14 Importantly, apoptosis was greater at 24 hours compared to 48 and 72 hours. This suggests that brain injury starts shortly after the hemorrhage and any treatment strategy to protect the brain should be instituted soon after hemorrhage. The cellular death in periventricular area of pups with IVH has perhaps multifactorial etiology and is possibly mediated by generation of cytokines, proteases, free radicals, and other immunomodulators.23,24 In addition to apoptosis, we noted evidence of axonal damage on APP and neurofilament immunostaining. To our knowledge, axonal damage in IVH has been evaluated neither in animal model nor in autopsy materials from premature infants. Despite the evidence of axonal damage, the only motor deficit noted was unsteady gait and poor ability to walk on inclination. This suggests that either injury was not severe enough to produce major signs of upper-motor-neuron lesion including hypertonia and paralysis, or more neurological deficit might appear later. Importantly, premature infants usually do not develop any motor weakness or spasticity shortly after IVH, similar to rabbit pups. The basis of axonal damage could be either plasma-lemmal disruption resulting from stretching due to ventricular dilation or apoptotic neuronal death/degeneration secondary to inflammatory mediators and oxidative stress or both.23,25

It is important to mention that we did not find any evidence of cerebral, pulmonary, or renal toxicity in the model. Of note, in rats, renal failure has been produced by intramuscular glycerol treatment by induction of myoglobinuria.26,27 Our protocol used intraperitoneal glycerol, a different route of administration that does not produce myoglobinuria and acute renal failure, as indicated by normal plasma creatinine, blood urea nitrogen and electrolytes in our glycerol treated pups. Glycerol itself is not toxic to tubular epithelium,28 but myoglobinuria and renal ischemia, a consequence of i.m. glycerol treatment, predispose to the development of acute tubular necrosis. Importantly, we did not find any evidence of direct neuronal toxicity induced by intraperitoneal glycerol as indicated by a lack of enhanced apoptosis and neuronal degeneration in glycerol-treated pups without gross IVH, similarly to saline-treated controls. Not surprisingly, intravenous glycerol therapy has been used to treat ischemic brain edema in humans without influencing cerebral glutamate, lactate, and pyruvate.29 Furthermore, glycerol is used to treat brain edema in brain tumors, trauma, and infection.30 Thus, the development of IVH in our rabbit model is not confounded by nephro- or neuro-toxicity because of direct effects of glycerol.

In conclusion, the present study describes standardized rabbit pup model of glycerol-induced GMH, in which forebrain (particularly the brain region around the ventricle) exhibits characteristics of inflammatory brain injury and cell death. The animal model does not show features of direct
glycerol-induced systemic toxicity. The model can potentially be used to evaluate therapeutic strategies both in prevention of GMH and for the treatment of posthemorrhagic complications.

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