Consequences of Intraventricular Hemorrhage in a Rabbit Pup Model

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Background and Purpose—Intraventricular hemorrhage (IVH) is a common complication of prematurity that results in neurological sequelae, including cerebral palsy, posthemorrhagic hydrocephalus, and cognitive deficits. Despite this, there is no standardized animal model exhibiting neurological consequences of IVH in prematurely delivered animals. We asked whether induction of moderate-to-severe IVH in premature rabbit pups would produce long-term sequelae of cerebral palsy, posthemorrhagic hydrocephalus, reduced myelination, and gliosis.

Methods—The premature rabbit pups, delivered by cesarean section, were treated with intraperitoneal glycerol at 2 hours postnatal age to induce IVH. The development of IVH was diagnosed by head ultrasound at 24 hours of age. Neurobehavioral, histological, and ultrastructural evaluation and diffusion tensor imaging studies were performed at 2 weeks of age.

Results—Although 25% of pups with IVH (IVH pups) developed motor impairment with hypertonia and 42% developed posthemorrhagic ventriculomegaly, pups without IVH (non-IVH) were unremarkable. Immunolabeling revealed reduced myelination in the white matter of IVH pups compared with saline- and glycerol-treated non-IVH controls. Reduced myelination was confirmed by Western blot analysis. There was evidence of gliosis in IVH pups. Ultrastructural studies in IVH pups showed that myelinated and unmyelinated fibers were relatively preserved except for focal axonal injury. Diffusion tensor imaging showed reduction in fractional anisotropy and white matter volume confirming white matter injury in IVH pups.

Conclusion—The rabbit pups with IVH displayed posthemorrhagic ventriculomegaly, gliosis, reduced myelination, and motor deficits, like humans. The study highlights an instructive animal model of the neurological consequences of IVH, which can be used to evaluate strategies in the prevention and treatment of posthemorrhagic complications. (Stroke. 2009;40:3369-3377.)

Key Words: germinal matrix hemorrhage–intraventricular hemorrhage • gliosis • myelin • ventriculomegaly • neurobehavioral testing • white matter

Germinal matrix hemorrhage–intraventricular hemorrhage (GMH-IVH) is a major problem of premature infants because both preterm birth rate and neonatal survival have substantially increased over the last 2 decades.1 The major neurological consequences of intraventricular hemorrhage (IVH) are cerebral palsy, posthemorrhagic hydrocephalus, and cognitive deficits.2 Because GMH-IVH is not preventable and since clinical treatments are inadequate, it is crucial to develop treatment strategies to prevent or minimize the neurological consequences. Therefore, a suitable animal model of the consequences of GMH-IVH is necessary to test new modalities of prevention and treatment.

GMH-IVH has been induced in several animal species, including the rat, mouse, dog, sheep, and pig, either by needle injection of blood into the ventricle or by altering hemodynamic parameters, including blood pressure, circulating blood volume, serum osmolarity, PCO₂, or O₂ levels.3,4 However, these animal models do not closely resemble premature infants with IVH with respect to etiology, pathology, and clinical consequences.5–7 Needle insertion in the brain for infusion of blood has an inherent disadvantage of producing direct injury to the brain and changing hemodynamic factors such as induction of hypercarbia, hypoxia, or hypervolemia confounds the IVH-induced brain pathology. A typical
perventricular white matter (WM) injury or hemorrhagic infarction, often noted in infants with IVH, does not develop in these models. The incidence of posthemorrhagic hydrocephalus in the blood infusion model is high relative to preterm neonates with IVH, and the animals do not exhibit motor impairments (hypertonic cerebral palsy) similar to premature infants.9-10 Of note, hypertonic cerebral palsy has not been produced in rodent pups; and full-term piglets are resistant to WM injury due to advanced neurological development. Another limitation of the existing models is failure to use prematurely delivered animals to model IVH because this requires special expertise to rear them. Hence, there is lack of an animal model of neurological consequences of IVH that naturally mimics preterm survivors of IVH.

We selected premature rabbit pups to model consequences of IVH because of their close resemblance to humans in several aspects.11 First, rabbit pups have a gyrencephalic brain, abundant germinal matrix, and perinatal brain growth, unlike rodents. Second, hypertonic cerebral palsy has been successfully produced in rabbit pups, but not in other species.12 Third, premature rabbit pups, like premature infants, are at risk of spontaneous germinal matrix hemorrhage (10%), which is substantially increased with intraperitoneal glycerol (80%) administration.11 Glycerol treatment results in dehydration and high serum osmolarity, which is attended by intracranial hypotension and selective rupture of the germinal matrix vasculature.14 Fourth, hemorrhage in this model, as we recently reported, leads to inflammatory changes around the lateral ventricle, just like in preterm infants.15 Fifth, the model is not confounded by significant toxicity of glycerol on the brain, kidney, lung, or other organs.13 Therefore, we chose to evaluate the neurological consequences of IVH in this rabbit pup model. We asked whether induction of moderate-to-severe IVH in the brain of premature rabbit pups would produce cerebral palsy and posthemorrhagic hydrocephalus and whether the neurological sequelae were associated with reduced myelination of WM, neuronal loss, and gliosis. In this study, we found that premature pups with IVH displayed posthemorrhagic ventriculomegaly, motor impairment with hypertonia, gliosis, and reduced myelination of WM, similar to humans.

Materials and Methods

Animal Experiment

The Institutional Animal Care and Use Committee of New York Medical College approved the animal protocol. The details of acute brain injuries in the first 3 days of life in our model of glycerol-induced IVH have been previously established and published.13 We obtained timed pregnant New Zealand rabbits from Charles River Laboratories, Inc, Wilmington, Mass. We delivered the pups prenatally by cesarean section at 29 days of gestational age (full-term, 32 days). Pups were immediately dried and kept in an infant incubator prewarmed to a temperature of 35°C. Pups were fed 1 mL rabbit milk at 4 hours of age and then approximately 2 mL every 12 hours (100 mL/kg per day) for the first 2 days using a 3.5-French feeding tube. After Day 2, we used kitten milk formula (KMR; PETAG Inc) and advanced feeds to 125, 150, 200, 250, and 280 mL/kg on postnatal Days 3, 5, 7, 10, and 14, respectively.

At 2 hours of age, the pups alternatively received 50% glycerol (6.5 g/kg) or saline treatment intraperitoneally. Head ultrasound was performed at 24 hours of age to assess for the presence and severity of IVH using an Acuson Sequoia C256 (Siemens) ultrasound machine. There was no difference in the presence and grading of IVH among 6, 24, and 72 hours of age in glycerol-treated pups on head ultrasound (selected samples). As described before, IVH was classified as (1) mild, no gross hemorrhage and hemorrhage detected on microscopy of hematoxylin and eosin-stained brain sections; (2) moderate, gross hemorrhage into lateral ventricles without significant ventricular enlargement (2 separate lateral ventricles discerned); or (3) severe, IVH with significant ventricular enlargement (fusion of ventricles into a common chamber) and/or intraparenchymal hemorrhage.14 Because microscopic IVH cannot be diagnosed by head ultrasound, we followed pups with moderate and severe IVH for a 2-week period to evaluate neurological consequences. We included both glycerol-treated and saline-treated non-IVH pups as controls.

Rabbit Tissue Collection and Processing

Tissue processing was done as described.13 The brain slices were immersion-fixed in 4% paraformaldehyde and cryoprotected into sucrose. Tissues were frozen into optimum cutting temperature compound (Sakura). Frozen coronal blocks were cut into 12-μm sections using a cryostat.

Immunohistochemistry and Nissl Staining, Quantification of Myelination, Gliosis, and Neuronal Density

We have described these in Supplemental Methods.

Western Blot Analyses and Electron Microscopy

The techniques are illustrated in Supplemental Methods.

Neurobehavioral Examination

We performed neurobehavioral testing at postnatal Day 14 based on a modification of neurobehavioral scoring protocol described elsewhere.12,13 The testing was performed by 2 blinded physicians. We evaluated cranial nerves by testing smell (aversive response to ethanol), sucking, and swallowing (formula was given with a plastic pipette). 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 scale), motor activity, locomotion at 30° angle, righting reflex, and gait. Tone was assessed by active flexion and extension of forelegs and hind legs (score 0 to 3). The righting reflex was evaluated by their ability and rapidity to turn prone when placed in a supine position. Sensory examination was limited to touch on the face (touching face with a cotton swab) and extremities as well as pain on limbs (mild pin prick). Grading of tone, gait, and locomotion at a 30° angle are described in the footnote of the Table. To assess coordination and muscle strength of extremities, we evaluated the ability of the pups to hold their position at 60° slope. The test was conducted on a rectangular surface (18×6 inch) placed at 60° inclination. We placed the pup at the upper end of the surface and measured the latency to slip down the slope. To assess vision, we performed a visual cliff test. The test scored whether pups stopped at the edge of an apparent cliff. All animals could detect the cliff.

Diffusion Tensor Imaging

Premature rabbit pups with glycerol-induced IVH and glycerol-treated non-IVH controls of 2 weeks postnatal age were anesthetized and transcardially perfused with 0.01 mol/L phosphate-buffered saline followed by 4% paraformaldehyde. The brains were harvested and immersion fixed in 4% paraformaldehyde. Before MRI, the brains were washed into phosphate-buffered saline and placed into a home-built MRI compatible tube. The tube was filled with Fluorinert (Sigma), an MRI susceptibility matching fluid. Imaging was conducted on a 9.4-T horizontal bore magnet (Bruker) with a custom-made cosine 1H radio frequency coil (14 mm diameter). The technical details of diffusion tensor imaging (DTI) experiments are described in Supplemental Methods.
Statistics and Analysis
We compared cross-sectional areas (ventricle, cortex, and forebrain), neuronal count, astrocyte density, ratio of myelinated to unmyelinated area, and myelin basic protein (MBP) levels between IVH pups and saline- as well as glycerol-treated non–IVH controls. We used the Mann–Whitney U test (nonparametric variables) or t test (parametric variable) to perform pairwise comparison and analysis of variance to compare multiple groups. A probability value of <0.05 was considered significant.

Results
Survival and Growth of Rabbit Pups
Because premature rabbit pups are not a common animal model and because they die with minor insult, we evaluated their survival in our laboratory. The pups were hand-fed because the dams were euthanized after cesarean section. Fifteen percent of the glycerol-treated IVH pups died within 72 hours and another 15% by Day 14 postnatal age (n=20), whereas 19.1% of glycerol-treated (n=15) and 18% of saline-treated (n=17) non–IVH controls died by Day 14. Among IVH pups, the cause of death was either episodes of prolonged seizures or aspiration of formula during feeding within the first 3 days. The deaths after 3 days in this group were attributed to an increase in intracranial pressure (neck retraction and opisthotonus) or aspiration of formula. Among non-IVH pups, feeding-related issues were the predominant cause of death. We next compared the weight of 4 groups of pups: glycerol-induced IVH, glycerol-treated non–IVH controls, saline-treated non–IVH controls, and full-term pups (Figure 1A). Full term pups were reared by the dam and preterm pups were hand-fed. The 4 groups of pups had comparable weight at each epoch. Together, IVH pups had a survival of approximately 70% at Day 14 compared with approximately 80% in non-IVH controls; and the weight of hand-fed premature pups was comparable to dam-fed term pups.

Clinical Consequences of IVH in Rabbit Pups
To determine the motor and sensory capabilities of IVH pups compared with non-IVH controls, we performed neurobehavioral examination at Day 14 (Table). We found weakness in extremities of 25% pups (n=20) with IVH: 3 pups with hind

Table. Neurobehavioral Evaluation of Premature Pups (E29) at Day 14 Postnatal Age

<table>
<thead>
<tr>
<th>System</th>
<th>Test</th>
<th>Glycerol (+)</th>
<th>Glycerol (+)</th>
<th>Glycerol (-)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n=20)</td>
<td>(n=17)</td>
<td>(n=15)</td>
<td></td>
</tr>
<tr>
<td>Cranial nerve</td>
<td>Aversive response to alcohol</td>
<td>3 (3, 3)</td>
<td>3 (3, 3)</td>
<td>3 (3, 3)</td>
</tr>
<tr>
<td></td>
<td>Sucking and swallowing</td>
<td>3 (3, 3)</td>
<td>3 (3, 3)</td>
<td>3 (3, 3)</td>
</tr>
<tr>
<td></td>
<td>Vision</td>
<td>3 (3, 3)</td>
<td>3 (3, 3)</td>
<td>3 (3, 3)</td>
</tr>
<tr>
<td>Motor</td>
<td>Motor activity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Head</td>
<td>3 (3, 3)</td>
<td>3 (3, 3)</td>
<td>3 (3, 3)</td>
</tr>
<tr>
<td></td>
<td>Forelegs</td>
<td>3 (2, 3)</td>
<td>3 (3, 3)</td>
<td>3 (3, 3)</td>
</tr>
<tr>
<td></td>
<td>Hind legs</td>
<td>3 (2.7, 3)</td>
<td>3 (3, 3)</td>
<td>3 (3, 3)</td>
</tr>
<tr>
<td></td>
<td>Righting reflex*</td>
<td>5 (4, 5)</td>
<td>5 (5, 5)</td>
<td>5 (5, 5)</td>
</tr>
<tr>
<td></td>
<td>Locomotion on 30° inclination†</td>
<td>3 (3, 3)</td>
<td>3 (3, 3)</td>
<td>3 (3, 3)</td>
</tr>
<tr>
<td></td>
<td>Tone‡</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
</tr>
<tr>
<td></td>
<td>Forelimb</td>
<td>0 (0, 25, 0)</td>
<td>0 (0, 0)</td>
<td>0 (0, 0)</td>
</tr>
<tr>
<td></td>
<td>Hind limb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hold their position at 60° inclination</td>
<td>6.7¶</td>
<td>12.1</td>
<td>11.5</td>
</tr>
<tr>
<td></td>
<td>(latency to slip down the slope in seconds)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Distance walked in 60 seconds, inches</td>
<td>61.5¶</td>
<td>104</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>Gait§</td>
<td>3 (2.75, 3)¶</td>
<td>4 (4, 4)</td>
<td>4 (4, 4)</td>
</tr>
<tr>
<td></td>
<td>Motor impairment</td>
<td>25%</td>
<td>0%</td>
<td>0%</td>
</tr>
<tr>
<td>Sensory</td>
<td>Facial touch</td>
<td>3 (3, 3)</td>
<td>3 (3, 3)</td>
<td>3 (3, 3)</td>
</tr>
<tr>
<td></td>
<td>Pain</td>
<td>3 (3, 3)</td>
<td>3 (3, 3)</td>
<td>3 (3, 3)</td>
</tr>
</tbody>
</table>

Values are median and interquartile range. Zero 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, does not walk; 1, takes a few steps (less than 8 inches); 2, walks for 9–18 inches; 3, walks very well beyond 18 inches.
‡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.
§Gait was graded as 0 (no locomotion), 1 (crawls with trunk touching the ground for few steps and then rolls over), 2 (walks taking alternate steps, trunk low and cannot walk on inclined surface), 3 (walks taking alternate steps, cannot propel its body using synchronously the hind legs, but walks on 30° inclined surface), 4 (walks, runs, and jumps without restriction, propels the body using synchronously the back legs, but limitation in speed, balance, and coordination manifesting as clumsiness in gait), or 5 (normal walking).
¶P<0.05, ¶P<0.001 for the comparison between pups with IVH and saline- as well as glycerol-treated non–IVH controls. Fisher exact test used to compare incidence of motor impairment and Mann Whitney U test for other parameters.
asymmetrical gait, walking with alternate steps, inability to walk. The scores for gait were significantly lower in IVH pups compared with controls \( (P<0.001) \). Furthermore, there was significant limitation in the speed of locomotion in IVH pups compared with controls \( (P<0.05) \). We also noted that scores of righting reflex were significantly lower in IVH pups compared with controls \( (P<0.05) \). The latency to slip down the slope was significantly reduced in IVH pups relative to controls \( (P<0.001) \). Of note, among pups with motor impairment, we observed slight increase in tone (score, 1) in the legs of 4 pups and considerable increase in tone (score, 2) in extremities of one pup. No visual or sensory impairment was found in any pup.

**Figure 1.** A, Growth chart of (1) premature pups with glycerol-induced IVH, saline-treated controls, and glycerol-treated controls (non-IVH); and (2) full-term pups. Data are mean±SEM \((n=8\) each). The 4 groups of premature pups had comparable weight. B, Healthy and paralyzed pup; note the difference in posture of 2 pups. The healthy pup (green label) is upright on the bench, whereas the paralyzed pup (red label) is unable to stand and is lying on the bench with stiff extremities. C, Coronal section through normal and IVH brain at 24 hours of age. Note ventricular dilation and fusion of the ventricles (block arrows) in IVH, whereas the normal brain has a silt-like ventricle (arrowhead). Scale bar=1 cm. D, Coronal section through the forebrain of a non-IVH pup at 2 weeks of age. Note the small ventricle (arrowhead). E, Coronal section through the forebrain of an IVH pup with ventriculomegaly (arrow) at 2 weeks of age. Scale bar=0.5 cm. F, Nissl staining of brain sections from a IVH (lower panel) and non-IVH (upper panel) pup. The sections were taken at 2 levels—midseptal nucleus (left panel) and ventrolateral nucleus of thalamus (right panel). Sections show ventricle, cortex, and WM.

leg weakness, one pup with predominantly foreleg weakness, and one pup with complete paralysis of both fore- and hind legs (Figure 1B). In contrast, non-IVH pups—saline- and glycerol-treated controls—did not manifest motor weakness in the extremities. The quadripareis and diplegia in pups were diagnosed by the presence of abnormal gait and limitation in the speed of walking. The gait in pups without motor weakness typically consisted of walking, jumping, and running with synchronous use of the hind legs. In contrast, pups with motor impairment presented with clumsiness in walking, asymmetrical gait, walking with alternate steps, inability to synchronously use the hind legs, or complete inability to walk. The scores for gait were significantly lower in IVH pups compared with controls \( (P<0.001) \). Furthermore, there was significant limitation in the speed of locomotion in IVH pups compared with controls \( (P<0.05) \). We also noted that scores of righting reflex were significantly lower in IVH pups compared with controls \( (P<0.05) \). The latency to slip down the slope was significantly reduced in IVH pups relative to controls \( (P<0.001) \). Of note, among pups with motor impairment, we observed slight increase in tone (score, 1) in the legs of 4 pups and considerable increase in tone (score, 2) in extremities of one pup. No visual or sensory impairment was found in any pup.

**Ventriculomegaly, Stretching, and Thinning of Cerebral Cortex, But No Cortical Atrophy in IVH Pups**

We performed gross and histopathologic evaluation of the brains at Day 14 (Figure 1C–F). The cross-sectional areas of the ventricle, whole forebrain, and cerebral cortex were measured in Nissl-stained brain sections (at 2 coronal levels: midseptal–nucleus and ventral posterolateral nucleus of the thalamus) of IVH and non-IVH pups. Data were plotted as box and whisker plots (Figure 2A–B). We found larger ventricular size in IVH pups compared with controls at both midseptal nucleus and thalamus levels \( (P<0.05\) each). Of note, cross-sectional area of the cerebral cortex or whole forebrain (excluding ventricles) was comparable between IVH pups and non-IVH controls.

We defined ventriculomegaly as a ventricular area that measures more than 3 SDs above the mean for age in non-IVH pups. Thus, at 2-week age, a ventricular area of ≥9 mm\(^2\) and 12.4 mm\(^2\) (mean±3 SD) at the level of midseptal nucleus and ventral posterolateral thalamus, respectively, was considered to be ventriculomegaly. Although 42% of IVH pups had ventriculomegaly (Figure 1E), none of the glycerol- and saline-treated controls had ventricular dilation. All the pups with ventriculomegaly exhibited palpable anterior fontanel and separation of cranial sutures, unlike controls. Predictably, all pups with severe IVH \( (n=3) \) developed ventriculomegaly, whereas only 22% of pups with moderate IVH had ventricular dilation \( (n=9) \).

We next measured cortical thickness and circumference of the brain section. We found significant reduction in cortical thickness in the forebrain of IVH pups (both with and without ventriculomegaly) compared with non-IVH controls (Supplemental Figure I, available online at http://stroke.ahajournals.org). The forebrain circumference was significantly greater in IVH pups with ventriculomegaly compared with non-IVH controls, but not in IVH pups without ventriculomegaly.

We next performed neuronal count in the cerebral cortex of IVH pups compared with glycerol- and saline-treated controls. Importantly, we found no significant difference in the neuronal density in IVH pups compared with non-IVH controls (data not shown). Together, IVH pups at Day 14 exhibited ventriculomegaly and thinning as well as stretching of the cortical mantle, but there was no evidence of cortical atrophy.
Reactive Gliosis in IVH
We immunolabeled coronal brain sections with glial fibrillary acidic protein antibody and performed astrocyte count in the periventricular zone, superficial WM (corona radiata and internal capsule), and cerebral cortex. We observed abundant hypertrophic astrocytes—with large cell body and numerous processes making a dense network—in the periventricular zone and WM of IVH pups in contrast to glycerol- and saline-treated non-IVH controls (Figure 2C–D). Accordingly, astrocyte count was significantly higher in the periventricular zone and WM of IVH pups compared with controls (P<0.05 each), but not in the cerebral cortex. Hence, IVH resulted in periventricular astrogliosis in premature pups.

Reduced Myelination in IVH
Because periventricular leukomalacia is associated with IVH, we next evaluated myelination in IVH pups compared with non-IVH controls. We double-labeled cryosections with MBP and pan-axonal filament-specific antibodies and measured ratio of myelinated (MBP) and unmyelinated fibers (pan-axonal filament, n=10). We found that the expression of MBP in the corona radiata, corpus callosum, and internal capsule was significantly lesser in IVH pups compared with both glycerol- and saline-treated controls (P<0.05 each; Figure 3A–B). Furthermore, MBP expression was similar in IVH pups with and without ventriculomegaly (n=5 each, data not shown). Of note, regional comparison within the WM areas revealed that MBP level was lower in the corona radiata and corpus callosum than in the internal capsule (P=0.016 and 0.014, respectively) in IVH pups. To further confirm our finding, we quantified MBP in the brain homogenates by Western blot analysis. Consistent with immunostaining data, we found that MBP protein level was significantly lower in IVH pups than controls (Figure 3C–D). In conclusion, development of IVH is attended by reduced expression of MBP.

Ultrastructural Studies
Ultrastructural evaluation did not show major differences in axonal and myelin morphology in the WM regions—corona radiata, corpus callosum, and internal capsule—of IVH pups compared with non-IVH controls at Day 14 (Figure 4). Overall, the myelinated and unmyelinated fibers were well organized and preserved in IVH pups, similar to non-IVH controls. However, a few axons in IVH pups showed features of axonal degeneration, including intra-axonal vacuoles and autophagosomes, which were not seen in non-IVH pups. We did not observe thinning of myelin, hypermyelination, Wallerian-like axonal degeneration, or the presence of inflammatory cells in IVH pups. In addition, remyelinating axons and very small axons representing regenerating sprouts were not identified.

DTI in IVH Pups
Because MRI is highly sensitive in quantifying WM injury, we performed ex vivo DTI on fixed brain of IVH pups and glycerol-treated non-IVH controls (n=4 each). We used maps of apparent diffusion coefficient (ADC) and fractional anisotropy (FA) to evaluate changes in the WM. ADC maps showed ventriculomegaly in IVH pups, whereas ventricles...
were slit-like in non-IVH controls (Figure 5A). Ventriculomegaly was bilateral and symmetrical in the lateral ventricles. FA, a directionally invariant index of diffusion anisotropy, depicts variance among the 3 eigenvalues of diffusion tensor. Directionally encoded color maps were used to reflect orientation-specific anisotropies in the medial–lateral, dorsal–ventral, and anterior–posterior directions with red, green, and blue colors, respectively. The WM region of interest, including the corona radiata, corpus callosum, internal capsule and fimbria–fornix, were evaluated (Figure 5B–E). In IVH pups, the FA was significantly decreased in the corpus callosum, corona radiata, and fimbria–fornix compared with controls ($P < 0.05$ each), but not in the internal capsule. The FA changes in the corpus callosum and fimbria–fornix were dominant in the medial–lateral direction, whereas FA was dominant in the dorsal–ventral direction within the corona radiata. As observed in Figure 5B, specific WM areas in the corpus callosum, fimbria–fornix, and corona radiata were significantly reduced in size in IVH pups compared with controls, but not in the internal capsule (Supplemental Table, available online at http://stroke.ahajournals.org).

**Figure 3.** Reduced myelination in IVH pups than non-IVH controls. A–B, Representative immunofluorescence of cryosections labeled with MBP and pan-axonal filament-specific antibodies. The ratio of myelinated to unmyelinated fibers was measured in the corpus callosum (CC), corona radiata (CR), and internal capsule (IC) using Metamorph software. Bar graph shows mean±SEM ($n=10$ each). Reduced myelination in IVH pups was noted in all the 3 WM regions—CR, CC, and IC—compared with glycerol- and saline-treated controls. Scale bar=20 μm. C–D, Representative Western blot analysis of MBP on brain homogenates. The bar graph shows mean±SEM ($n=6$ pups). MBP levels were normalized to β-actin levels. Reduced myelination in IVH pups was noted relative to saline- and glycerol-treated non-IVH controls. There was no statistical difference between saline and glycerol controls. *$P<0.05$ for IVH pups versus glycerol-treated non-IVH pups. #$P<0.05$ for IVH pups versus saline-treated non-IVH pups.

**Discussion**

GMH-IVH continues to be a major problem of modern neonatal intensive care units worldwide. In this study, we evaluated the neurological consequences of IVH in premature rabbit pups, in which IVH was induced by intraperitoneal glycerol at 2 hours postnatal age. We found that premature pups with IVH developed motor impairment with hypertonia (25%) and ventriculomegaly (42%) at 2 weeks postnatal age. Accordingly, the pups showed histological and radiological evidence of reduced myelination and gliosis. The study underscores a novel animal model that can be used to evaluate strategies in the prevention and treatment of the consequences of IVH.

We have recently reported characterization of acute brain injury (<72 hours) in our rabbit pup model of IVH; and here, we are describing relatively long-term outcomes (2-week) of IVH in rabbit pups. Our model mimics human conditions and has a number of merits. First, development and progression of IVH in this model is morphologically similar to IVH in premature infants because hemorrhage initiated by rupture of germinal matrix vasculature progresses to IVH. Second, induction of IVH in our model neither causes direct injury to the brain by needle stab nor confounds the...
model with unwanted metabolic changes in the neural cells by hypoxia—ischemia or hypercapnia, as used in several studies. Third, there are a number of inherent benefits of using the rabbit that have been described in the introduction. Fourth, rabbit pups with IVH displayed neurological complications of motor impairment, ventricular dilation, and reduced myelination, just like in premature infants. However, there are some limitations of our model. We delivered pups by cesarean section, euthanized the dams, and nursed orphan pups in an infant incubator. These pups were hand-fed using feeding tubes, which was labor-intensive requiring technical expertise and experience. Although glycerol does not produce major systemic adverse effects, this can potentially open the blood–brain barrier and exert metabolic changes in the brain, similarly to mannitol. Together, our model of neurological consequences of IVH in premature rabbit pups mimics premature infants with posthemorrhagic complications.

To our knowledge, this is the first animal model depicting neurological consequences of IVH in a prematurely delivered animal. Because our rabbit pups (E29) are 3 to 4 days premature (term, 32 days; E29, 87% to 90% gestation), they are equivalent to premature infants of approximately 33-week gestational age. However, on linking cortical and noncortical development of rabbits with humans, the neurodevelopment of E29 rabbits equates to 18 weeks and P11 rabbits (postnatal Day 14 for E29 pups) to 29 weeks of gestational age in humans. Furthermore, myelination begins at postnatal day 4 to 7 in term rabbit pups and in the second trimester of pregnancy in humans. Based on these considerations, our Day 14 pups are comparable to premature human infants of 29 to 35 weeks’ gestation; and postnatal care of a 2-week duration provided to E29 rabbits is equivalent to 14 to 18 weeks of neonatal care given to premature infants. However, these comparisons have obvious limitations because human neurodevelopment is more complex and intricate compared with those of rabbits or rodents.

The most important and novel observation made in the model was that premature pups displayed clinical evidence of hypertonia with motor impairment and ventriculomegaly, similar to premature infants. We found that 25% pups with IVH exhibited signs of cerebral palsy, including hypertonia, abnormal gait, limitation in locomotion, and poor ability to hold their position at 60° inclination without slipping. We observed only a slight increase in tone among pups with motor impairment, except for one pup that showed considerable increase in tone. Consistent with our findings, several studies have shown that IVH in premature infants have a higher occurrence of cerebral palsy and other neurological sequelae compared with premature infants without IVH.

A prospective study has reported that 24% of infants with Grade III (moderate) and IV (severe) IVH have abnormal neurological diagnoses, including cerebral palsy and cognitive deficits at 5 year of age. Importantly, premature infants with IVH or other brain injuries, who sustain WM damage, may not manifest with significant spasticity or other signs of cerebral palsy immediately at birth; however, neurological manifestations may appear at later age and progress over weeks or months. In contrast to motor impairment, we did

Figure 5. DTI of rabbit pup brain. A, Coronal ADC maps at the level of midseptal nucleus and ventral posterolateral nucleus of the thalamus show larger ventricles in IVH pup and glycerol-treated controls. B, FA maps of contiguous coronal slices of pups (Day 14) with IVH and without IVH. WM structures—corpus callosum (CC), corona radiata (CR), and fimbria fornix (FF)—show decreased FA in IVH pups compared with glycerol-treated control. C–E, FA in CC, CR, and FF, respectively, for the dominant orientation coordinates of medial–lateral (R), dorsal–ventral (G), and anterior–posterior (B) directions. FA was significantly decreased in the CC ($P < 0.05$), CR ($P < 0.03$) and FF ($P < 0.05$) in IVH pups compared with glycerol-treated controls. The FA changes in CC and FF were dominant in the medial–lateral direction, whereas FA was dominant in the dorsal–ventral direction within CR.
not observe any apparent sensory involvement in preterm rabbits, just like preterm infants.\(^\text{20}\) Another important clinical manifestation in our model was the development of posthemorrhagic ventricular dilation in 42% of IVH pups, but not in controls. In a neonatal rat model of hydrocephalus, 65% of pups (P7) injected with blood into the cerebral ventricle developed hydrocephalus, whereas 50% of pups injected with artificial cerebrospinal fluid also developed hydrocephalus.\(^\text{10}\) However, clinical studies in premature infants with IVH have reported hydrocephalus in 9%, 36%, and 47% survivors of mild, moderate, and severe IVH, respectively, similar to our animal model.\(^\text{9}\) We also noted reduced cortical thickness and increased circumference of the forebrain of IVH pups compared with saline- and glycerol-treated non-IVH controls. However, cerebral cortical area was comparable between IVH pups and non-IVH controls. This suggests stretching and thinning of cortical mantle, yet preserving the cross-sectional cortical area in IVH pups. Together, motor deficits and ventriculomegaly in our model mimic premature infants with IVH in a number of aspects, suggesting the clinical relevance of the model.

Another key observation was reduced myelination in IVH pups relative to non-IVH controls. In addition, there was no difference in myelination between IVH pups with and without hydrocephalus. Consistent with the findings in our model, an association between IVH and WM injury in premature infants has been reported by many investigators.\(^\text{21,22}\) Notably, in one old series of premature infants who died with IVH, periventricular leukomalacia of some degree has been observed in 75% cases.\(^\text{7}\) The possible mechanisms of underlying WM injury in IVH include: (1) destruction of germinal matrix and periventricular WM; (2) concomitant reperfusion injury of the ischemic region around the area of hemorrhage; (3) neutrophil and macrophage infiltration as well as apoptosis of neural cells;\(^\text{13}\) and (4) posthemorrhagic hydrocephalus.\(^\text{2}\) Importantly, IVH pups exhibited preservation of myelinated and unmyelinated fibers except for focal axonal degeneration (Figure 4). Our previous study on acute brain injuries in IVH has revealed evidence of axonal damage in the pups with IVH during the first 48 hours of life.\(^\text{13}\) Thus, it seems likely that most of the pathological changes in axonal morphology were transient and were repaired over the next 2-week period. In conclusion, the present animal model of IVH, exhibiting hypomyelination without major axonal degeneration, is similar to preterm survivors of IVH.\(^\text{25}\)

We used DTI to quantify and visualize WM patterns in IVH pups compared with non-IVH controls. Consistent with histological data and Western blot analyses, we found reduced FA in the corpus callosum, fimbria-fornix, and corona radiata of IVH pups compared with non-IVH controls. However, FA and area of the internal capsule in IVH pups was similar to non-IVH controls. It seems that the internal capsule sustained smaller damage than the corona radiata and corpus callosum because the internal capsule is located at a greater distance from the ventricle compared with the corona radiata and corpus callosum. Indeed, immunohistochemistry revealed lesser myelination in the corona radiata and corpus callosum than the internal capsule in IVH pups (Figure 3). Of note, ex vivo MRI on fixed brain provides very high spatial resolution (eg, 78×94×94 μm) because of high signal-to-noise ratio arising from time available for signal averaging.\(^\text{26}\) Other advantages of MRI are that it is 3-dimensional, free of tissue distortion and sectioning artifacts, and that it complements histological immunostaining methods. Together, our MRI study has established applicability of performing DTI in our model for further mechanistic and therapeutic studies.

In conclusion, the present study describes a novel rabbit pup model of neurological consequences of GMH-IVH, which displays manifestations of motor deficits and ventricular dilation, similar to human premature infants. In accordance with the clinical features, histological analysis revealed reduced myelination of the forebrain, which was further supported by DTI. This model seems to be an attractive tool for evaluating therapeutic strategies in the prevention and treatment of posthemorrhagic complications in premature infants.

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**Disclosures**
None.

**References**


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