Background and Purpose: Delayed cell loss in neonates after cerebral hypoxic-ischemic injury (HII) is believed to be a major cause of cerebral palsy. In this study, we used radiolabeled annexin V, a marker of delayed cell loss (apoptosis), to image neonatal rabbits suffering from HII.

Methods—Twenty-two neonatal New Zealand White rabbits had ligation of the right common carotid artery with reduction of inspired oxygen concentration to induce HII. Experimental animals (n=17) were exposed to hypoxia until an ipsilateral hemispheric decrease in the average diffusion coefficient occurred. After reversal of hypoxia and normalization of average diffusion coefficient values, experimental animals were injected with $^{99m}$Tc annexin V. Radionuclide images were recorded 2 hours later.

Results—Experimental animals showed no MR evidence of blood-brain barrier breakdown or perfusion abnormalities after hypoxia. Annexin images demonstrated multifocal brain uptake in both hemispheres of experimental but not control animals. Histology of the brains from experimental animals demonstrated scattered pyknotic cortical and hippocampal neurons with cytoplasmic vacuolization of glial cells without evidence of apoptotic nuclei by terminal deoxynucleotidyl transferase-mediating end-labeling (TUNEL) staining. Double staining with markers of cell type and exogenous annexin V revealed that annexin V was localized in the cytoplasm of scattered neurons and astrocytes in experimental and, less commonly, control brains in the presence of an intact blood-brain barrier.

Conclusions—Apoptosis may develop after HII even in brains that appear normal on diffusion-weighted and perfusion MR. These data suggest a role of radiolabeled annexin V screening of neonates at risk for the development of cerebral palsy. (Stroke. 2000;32:2692-2700.)

Key Words: apoptosis ■ brain injuries ■ hypoxia ■ newborn ■ radioisotopes ■ rabbits

Relatively minor fluctuations in the cerebral microcirculation can trigger programmed cell death (apoptosis) in neonates after a hypoxic-ischemic insult (HII).1-5 Unlike the immediate irreversible damage caused by severe prolonged ischemia (necrosis and infarction), apoptosis occurs over a period of time and may be the primary mode of delayed neuronal death in neonates. This type of cell death may be the precursor of cerebral palsy.6 Because apoptosis is a multistep process, with the lag phase lasting a significant amount of time, it is possible that agents that block the apoptotic cascade may inhibit the completion of programmed cell death, potentially saving injured cells.5-8 To determine whether such therapy is warranted, an in vivo noninvasive imaging marker that identifies cells committed to apoptosis is necessary.

See Editorial Comment, page 2699

Efforts to image neonatal HII have largely centered on MR techniques, including phosphorus ($^{31}$P) and (lipid/lactate) proton ($^1$H) spectroscopy, diffusion-weighted imaging (DWI), and gadolinium (Gd)-diethylenetriamine pentaacetic acid (DTPA) bolus tracking experiments.2,9-12 In a neonatal porcine model, Mehmet et al10 demonstrated high-energy phosphate depletion in the cingulate sulci after HII with $^{31}$P MR spectroscopy. High-energy phosphate loss was also directly correlated with the number of apoptotic hippocampal neurons in brains, without evidence of necrotic damage 48 hours after the insult.

DWI can identify small changes in the apparent diffusion coefficient (ADC), an indicator of regional diffusion of water...
molecules, which is a sensitive marker of the earliest metabolic effects of cerebral ischemia. Normal ADC values or timely normalization of ADC values, after brief periods of ischemia, suggest an absence of significant cerebral injury, particularly if found in conjunction with normal cerebral perfusion and an intact blood-brain barrier (BBB), as observed on MR bolus tracking and post–T1-weighted post–Gd-DTPA imaging experiments. However, the presence or absence of apoptosis with respect to diffusion-weighted MRI has been examined only in animals that suffered permanent ADC changes that directly correspond to areas of infarction induced by transient but moderately severe ischemia.

Although changes in diffusion correlate with apoptosis, they are not a direct marker of the process. In 1998, a radiopharmaceutical approach to detect apoptosis in vivo was described. The technique used 99mTc-labeled annexin V, which binds to phosphatidylserine (PS) on the outer leaflet of the cell membrane of cells undergoing apoptosis, which immediately follows caspase-3 activation. The technique has been validated in cell culture, in vivo studies of Fas receptor–mediated hepatic apoptosis, and during acute rejection of transplanted hearts, lungs, and livers. Because neurons also express PS as they undergo apoptosis, we hypothesized that annexin imaging could be useful in identifying this process in the brain of the neonate.

In the present study, we tested the ability of 99mTc-labeled annexin V to detect cerebral expression of PS in response to transient microcircular disturbances as defined by DWI and Gd-DTPA MR imaging during induction of neonatal HII. For the present study, we used a well-described rabbit model of neonatal HII in which a single common carotid artery was ligated, followed by lowering FIO2 to 10%. This model produces a global HII after an initial period of ipsilateral ischemic changes.

Materials and Methods

Neonatal Rabbit Model

After 8- to 10-day-old New Zealand White rabbits (n=22, 17 experimental and 5 control rabbits) were anesthetized with halothane, their right common carotid arteries were ligated. Either the right external jugular or a femoral vein was cannulated for infusion of the MR contrast agent and radiopharmaceuticals.

Experimental animals (n=17) were divided into 2 test groups: (1) The chronic group (9 animals) was subjected to 2 hours of hypoxia and 10 to 15 hours of reperfusion before annexin V injection. After surgery, the animals were transferred to a warm (37°C) hypoxic chamber (10% FIO2) and subjected to hypoxia for a total of 2 hours. These animals recovered overnight. Ten to 15 hours later, they were scanned by MRI, followed by injection of 99mTc annexin V and radionuclide imaging 2 hours later. (2) The acute group (8 animals) was subjected to 2 hours of hypoxia and 2 hours of reperfusion before annexin V injection. After surgical preparation, the animals were directly subjected to hypoxia and MRI as described below.

All animals were positioned in a 2.0-T GE Omega MR system and scanned by MRI bolus tracking and post–T1-weighted post–Gd-DTPA MR imaging during induction of neonatal HII. For the acute group, continuous monitoring of the metabolic status of the brain throughout the entire experimental period (baseline to recovery) was performed with the use of serial DWI to detect the onset of decreased signal intensity. Diffusion-weighted images (b=1300 s/mm2) and T2-weighted images (echo time 50 ms) were acquired at baseline (prehypoxia), just before the end of the HII period, and after hypoxia. These images were processed to yield ADC maps. After baseline images, the animals were exposed to hypoxia (10% FIO2) until there was decreased signal intensity throughout the entire ipsilateral hemisphere. Hypoxia was reversed immediately thereafter, and the animals recovered in 100% oxygen until diffusion-weighted hypointensity had resolved. Note that the decreases in ADC, once they appeared, spread quite rapidly, making it difficult to stop the insult at a point in time at which the decreased signal intensity was limited to just a single hemisphere. Therefore, there was a variable degree of overshoot of decreased ADC to the contralateral side.

Animals in the chronic group were not imaged acutely with MRI but were instead imaged with MRI 10 to 15 hours after HII and before annexin V injection. Prehypoxia and posthypoxia Gd-DTPA–enhanced T1-weighted MR images were acquired with the following parameters: repetition time 500 ms, echo time 12 ms, 2 excitations, 128x128 matrix, field of view 50 mm, and slice thickness 2.5 mm.

MRI Data Processing

Diffusion-weighted images were processed by using customized image display software at each scan time point (MR Vision Co). A 2-point fit was performed on the signal intensity decay curves of the baseline (ie, zero diffusion-weighted) images (M0, with b=0) and diffusion-weighted images (M, with b=1300 s/mm2). ADC was calculated from these 2 images according to the following:

\[ ADC = \frac{-\log(M/M_0)}{b}, \]

where \( b = gGd^2 / (\Delta - \delta g) \), G is the diffusion gradient strength, \( \delta \) is the duration of the rectangular shaped diffusion-weighting gradient pulses, and \( \Delta \) is the time between the leading edges of the diffusion gradient pulses.

Regions of interest (ROIs) were drawn in the uninvolved (contralateral) and ipsilateral brain by using the ROI tool of the image display software. The change in ADC was calculated as a percentage of the baseline value.

Radiopharmaceutical Preparation and Administration

99mTc-HYNIC annexin V was prepared as previously described. Briefly, human annexin V was produced by expression in Escherichia coli. Annexin V was conjugated with HYNIC, a bifunctional linker molecule with one moiety that binds to a protein lysine residue and another that binds to complexed of 99mTc. HYNIC-labeled annexin V was stored at -70°C until use. 99mTc was bound to HYNIC-labeled annexin V after reduction in a tin-tricine solution. Specific activity ranged from 100 to 200 μCi/μg protein, with a radiopurity of 92% to 97%.

Annexin V (2 to 4 mCi, 50 to 100 μCi/kg protein per animal) was administered intravenously 2 hours (acutely) and 10 to 15 hours (chronically) after HII. Three chronic test and 2 ligation control animals were cocultured with 200 μCi of 99mTc-DTPA to assess for the integrity of the BBB by use of a radiopharmaceutical technique.
Radionuclide Imaging
A mobile gamma camera (model 420, Technicare) equipped with a 1-mm pinhole collimator was used to record the radionuclide distribution. Images were recorded 2 hours after tracer administration. The animals were sedated with 80 mg/kg ketamine, administered intramuscularly before imaging.

The brain was imaged in the vertex (posterior) and right lateral positions for 20 minutes per view. Data were recorded in a dedicated system (ICON, Siemens) in a 256×256 matrix. The camera was set to image the 140-keV photopeak of 111In with a 20% window. In the animals coinjected with 111In-DTPA, 20-minute 256×256 acquisitions were performed with use of the same projections (without repositioning of brain) as described above. The pulse height analyzer was set to include both photopeaks of 111In.

Radionuclide Data Processing and Statistical Analysis
Images were analyzed by placing an ROI over normal areas of the brain and over zones of high uptake. The normal zones corresponded to regions of low cerebral activity (ie, cerebral tissue background [CTB]). Data were expressed as CTB (cpm) per number of pixels.

Regions of highest uptake (RoH) were recorded as counts (cpm) per pixel and then normalized to CTB as follows: RoH (cpm/pixel)/CTB (cpm/pixel).

The normalized activities obtained (see above) were averaged and presented as mean±SD for individual brain tissue regions. The cerebellar uptakes in the control and experimental groups were expressed as cerebellar counts (cpm) per pixel divided by CTB (cpm/pixel). Statistical comparisons between control and experimental mean values were performed by a 2-tailed Student t test for significance. A value of \( P \leq 0.05 \) was considered to be significant. Note that the intrasubject regional variations of annexin uptake within control brains (n=5 ligated, n=5 nonligated) were <29% for images taken in the posterior projection and 14% for those taken in the right lateral projection.

Histopathologic Analysis/In Situ Detection of Apoptotic Nuclei
Brains were excised and immediately put into PBS before radionuclide imaging (≈1.5 hours for each animal). Formalin fixation in situ was not performed because this may have interfered with annexin V binding. After annexin V imaging, the brains were transferred directly to phosphate-buffered formalin. Formalin-fixed paraffin-embedded tissues were sectioned coronally in 5 equally spaced locations in the cephalocaudal direction of the neonatal cerebral and cerebellum. Sections (5 \( \mu \)m) were then obtained and stained with hematoxylin and eosin. For the detection of apoptotic nuclei, corresponding 5-\( \mu \)m sections were stained by direct immunoperoxidase detection of digoxigenin-labeled 3'OH DNA strand breaks by use of the terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) method. The procedures used were outlined in the commercially available Apop Tag Kit (Oncom Inc).

Hematoxylin and eosin–stained and TUNEL-stained sections were examined for regions of ischemic damage and the presence of apoptotic nuclei (TUNEL-positive nuclei with clumped chromatin).

Immunohistochemical Staining of Intravenously Injected Biotin-Annexin
Subgroups of animals were as follows: acute hypoxia (n=2), chronic hypoxia (n=2), and control (n=2); all animals were coinjected with biotin-labeled annexin V (300 \( \mu \)g/kg protein, Molecular Probes) and radiolabeled annexin V. Two hours after coinjection, ex vivo brain specimens were imaged and then flash-frozen on dry ice (≈20°C) without formalin fixation. Frozen histological sections (20 \( \mu \)m) were obtained in the coronal plane, including the cortex and midbrain of each animal. These sections were then fixed with 75% acetone/25% ethanol, washed with 0.003% hydrogen peroxide, digested for 15 minutes with proteinase K solution, and placed in streptavidin-conjugated horseradish peroxidase PBS bath for 40 minutes. Sigma Fast DAB (tablets) solution was applied for 10 minutes, followed by quenching. After identifying cells positive for annexin V–biotin (brown stain), sections were then colabeled with cell-type markers to identify neurons and astrocytes. Sections were treated with 0.5% Triton X-100 for 20 minutes and then blocked in 5% normal serum. Primary antibodies to identify neurons (MAP2B antibody, 1:100 dilution; M41420, Transduction Laboratories) or astrocytes (GFAP antibody cocktail, 1:200 dilution; 60341D, Pharmingen International) were applied at room temperature for 1 hour. Sections were then incubated in biotinylated secondary antibodies, followed by an alkaline phosphatase–based avidin-biotin complex and then Vector Blue as the chromogen (all reagents were purchased from Vector Laboratories).

Results
MR Imaging
Animals in the acute group showed decreased ADC throughout the ipsilateral cortex and subcortical white matter and a variable degree of overshoot to the contralateral cortex at peak hypoxia. In all animals, these decreases resolved completely after reversal of hypoxia during recovery. The regions of focally increased annexin V uptake were primarily in the frontal/frontoparietal region and midbrain, which did not precisely match regions of ADC change despite some clear regions of overlap. Note that no MRI was performed on the chronic group during the hypoxic interval.

In the acute experimental animals, decreased ADC in the ipsilateral hemisphere was ≈50% of baseline (range 46% to 55%), and there was essentially no change in the contralateral uninvolved brain tissue. All brains, control and experimental, showed no areas of decreased ADC in the DWI images before radionuclide injection. T1-weighted images also showed no contrast leakage before annexin injection V, as shown in Figure 1.

The animal in Figure 1 showed focally increased annexin V uptake primarily in the ipsilateral hemispheric, bifrontal,
midbrain and basilar regions. These areas of uptake did not precisely match those seen in the ADC images, although there was some degree of overlap.

\[ \text{\textsuperscript{99mTc} Annexin V Radionuclide Imaging} \]

Normal control animals (n = 7) did not show any regions of focally increased annexin V uptake on in vivo or ex vivo posterior and right lateral views. Ex vivo imaging of ligated control animals (n = 5) showed a single animal with a focal region of annexin V uptake in the frontal parietal junctional area (uptake on posterior view, 1.95; right lateral view, 2.59). Nonligated control brains (n = 2) also did not demonstrate any regions of focally increased annexin V uptake ex vivo. Ex vivo images of control animals showed slightly (10% to 14%) higher baseline counts per pixel in the cerebellar tissue compared with the rest of the brain. The average cerebellar uptake in control animals was 1.104 \pm 0.129 in the posterior views and 1.136 \pm 0.143 in the right lateral views. Figure 2 shows the typical in vivo/ex vivo annexin V distribution in the ligation/control group. Ex vivo \textsuperscript{111}In-DTPA images of ligated control animals demonstrated no regions of increased uptake (n = 2) (data not shown).

In vivo (Figure 3A and 3B) and ex vivo (Figure 3C and 3D) imaging of the hypoxic-ischemic animals (n = 17) all showed focally increased annexin V uptake. The small size of these animals precluded single-photon emission CT radionuclide imaging. Given these circumstances, it was not possible to subtract the expected normal background calvarial bone marrow and soft tissue uptake\textsuperscript{28} from brain uptake in vivo. Therefore, the ex vivo data were used for ROI analysis. ROI analysis of all ex vivo posterior images demonstrated that the frequency of focally increased annexin V uptake in brain regions in order of occurrence was as follows: in the posterior views, cerebellum > frontalbrain region > parietal junction. The frequency of abnormal focal annexin V uptake in the right lateral ex vivo views was as follows: cerebellum > frontalbrain region > midbrain > frontalparietal junction > basilar and occipitoparietal junction.

ROI analysis of the acute and chronic groups showed abnormally increased focal cerebellar annexin V uptake, which was significantly greater than that of the control group. In the acute group, cerebellar uptake in the posterior views was 1.582 \pm 0.388 (P < 0.025); in the right lateral views, uptake was 1.772 \pm 0.762 (P = 0.08, borderline significance). In the chronic group, cerebellar uptake in the posterior views was 2.029 \pm 1.086 (P < 0.005), and in the right lateral views, uptake was 2.197 \pm 0.938 (P < 0.005).

The cerebral brain regions of the acute group showed fewer foci of abnormally increased annexin V uptake compared with those of the chronic group (8 cerebral regions in the acute group versus 20 cerebral regions in the chronic group). Figure 4 shows a scatterplot of the distribution of these focal regions among the entire experimental population.

A subset of the chronic experimental group cojected with \textsuperscript{111}In-DTPA (n = 3) demonstrated no focal uptake in the cerebrum or cerebellum, indicating an intact BBB (data not shown).

\[ \text{Histopathological Findings} \]

Histological examination of formalin-fixed experimental brains (n = 13) showed patchy ischemic changes in the following tissues: cortex, CA1, and CA3/4. Vacuolar changes were frequently seen in the periventricular white matter (Figure 5A), with scattered pyknosis of the neurons (Figure 5B). These ischemic changes were generally more pronounced on the right side of the brain, the side of ligation. The formalin-fixed brains from control animals showed no patho-
logical change in hematoxylin and eosin–stained sections (n=5). TUNEL staining was negative for all formalin-fixed brains (18 total).

**Immunostaining for Neurons, Astrocytes, and Injected Biotinylated Annexin V**

Double labeling of intravenously administered biotinylated annexin V cerebral deposition and the neuronal marker, MAP2B, showed scattered neurons (few per ×40 field) positive for both exogenous annexin V and MAP2B (see Figure 6A) in all groups of animals examined (ie, control, acute, and chronic hypoxic groups). However, qualitatively, there were many more double-staining neurons in both groups of hypoxic animals compared with control animals, which demonstrated little double staining. Double labeling of biotin–annexin V deposition and the astrocyte marker, GFAP, showed single staining of scattered annexin V–positive cells (few per ×40 field) with a triangular (neuronal) morphology in all groups (see Figure 6B). Annexin V staining was also rarely observed in the cytoplasm of GFAP-positive cells (ie, astrocytes).

**Discussion**

Our results demonstrate that there is selective multifocal localization of radiolabeled annexin V in both hemispheres of neonatal rabbits subjected to global HII. Furthermore, abnormally increased annexin V uptake occurred in brains that had fully recovered their normal energy state after reversal of global hypoxia. These results suggest that the process of apoptosis maybe triggered in tissues that have recovered their normal energy state (as seen by diffusion-weighted MRI) or that had no detectable (at least in this experimental system) ADC changes during hypoxia. In the clinical setting, a neonate may also develop cerebral palsy because of delayed cell loss after relatively minor degrees of HII.1,6

The degree of HII that experimental animals in the present study underwent was relatively mild, as indicated by the absence of permanent changes in cerebral ADC, perfusion, or loss of BBB integrity. Brains that show no abnormalities of water diffusion, cerebral perfusion, or BBB breakdown (shown by MR and 111In-DTPA imaging) after reversal of hypoxia would be expected to have little or no uptake of radiolabeled annexin V, particularly in the chronic group.9 However, abnormal increases in annexin V uptake were seen bilaterally in a distinctly different pattern, although not totally dissimilar, compared with that of the transient hemispheric ADC and perfusion abnormalities observed on MR. The single exception was one ligation control animal with a focus of increased annexin V uptake in the frontoparietal region. Whereas MRI showed no diffusion-weighted abnormalities in this animal, hypoxic-ischemic damage during delivery or during carotid artery ligation could not be excluded histologically because the brains from this subgroup of control animals were frozen before fixation for immunolabeling, precluding accurate histological assessment of the presence of subtle morphological changes.

Histological analysis of the formalin-fixed brains of experimental animals demonstrated subtle but consistent ischemic changes scattered throughout the cortex, hippocampus, and periventricular white matter in both hemispheres that were not observed in ligated or nonligated control animals. The association of vacuolar changes in the cytoplasm of periventricular glial cells seen with HII may represent cytoplasmic lipid droplets and vesicles that are leached out by the organic solutions used to fix and prepare histological sections. Cytoplasmic lipid droplets (1.08 μm in average diameter) have been observed in thymocytes and glial tissues/tumors undergoing apoptotic cell death in response to therapy.29–31 These droplets are also observable in vivo by 1H lipid MR spectroscopy. It appears that both MR spectroscopy and radiolabeled annexin V radionuclide imaging maybe useful for the detection and monitoring of the early molecular events of apoptosis that occur before end-stage irreversible autocleavage of nuclear DNA.

TUNEL immunohistochemical staining of formalin-fixed brains, a marker of autodigested DNA in situ, was negative.
for the neurons and glial cells, which showed subtle but real morphological changes after reversal of hypoxia. Previous investigations by Du et al\(^3\) and Mehmet et al\(^{10}\) failed to demonstrate apoptotic nuclei by in situ TUNEL immunohistochemical staining by 48 hours after the reversal of relatively mild (not immediately necrotic) degrees of HII. However, exposure of PS on apoptotic cells, which bind annexin V in vivo, occurs much earlier, before the autodigestion of DNA that can be detected by TUNEL staining or gel electrophoresis.\(^{17,32-34}\) In addition, PS exposure serves as a signal to adjacent cells and phagocytes that an apoptotic neuron or glial cell is ready for engulfment and ingestion.\(^{18,35}\) The combination of these factors and perhaps others may help to explain the relatively few TUNEL-positive neurons noted several days after HII in prior studies and their absence before 24 hours in the present study.

In frozen brain tissue, the specific cellular localization of radiolabeled annexin V appeared to be within the neuronal cytoplasm (and rarely astrocytes) on the basis of staining for biotin–annexin V deposition. Interestingly, the BBB was noted to be functionally intact in the Gd-DTPA MR and \(^{111}\)In-DTPA radionuclide images. The ability of annexin V, a protein that is half the weight of albumin, to cross the BBB suggests an active mechanism of annexin V uptake that is part of neuronal and astrocytic physiology. Annexin V–positive neurons in control animals, although less common than in the experimental group, were unexpected and may be due to baseline rates of neuronal annexin V uptake involved in the physiological cell turnover (apoptosis) that is characteristic of normal neonatal brain development.\(^{36-38}\)

The cytoplasmic uptake of annexin V is unlikely to be explained by an artifact from the sectioning of the brains.
cerebellum is a major target for hypoxic damage in neonates and contributes to the pathophysiology of cerebral palsy. Purkinje cells normally demonstrate significantly increased amounts of PS during the first week after birth. This observation mostly likely is related to the marked amount of apoptosis of neurons in the external granular layer of the cerebellum noted in the neonatal period of development. On the basis of our data, further increases in PS expression occur with global hypoxia, presumably from the acceleration of apoptosis above the expected rates of cell death attributable to normal cerebellar maturation. However, we did not directly confirm the presence of annexin V localization in cerebellar neurons (or glial cells) because frozen cerebellar specimens were not specifically analyzed for the presence of intravenously administered biotinylated annexin V.

In summary, these experiments suggest that 99mTc-radiolabeled annexin V imaging is useful in identifying neonates acutely suffering from HII. In the future, this and other diagnostic imaging tools, such as 1H lipid MR spectroscopy, may prompt earlier administration of neuroprotective agents. Novel neuroprotective interventions undertaken in the acute post-HII situation may ultimately help to prevent or ameliorate neuronal and white matter loss associated with the development of cerebral palsy in preterm and term neonates.

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multifocal localization of radiolabeled annexin V in both hemispheres of these neonatal rabbits subjected to global hypoxic/ischemic injury. Abnormally increased annexin V uptake occurred in brains that had fully recovered their normal energy state after reversal of global hypoxia. The authors indicate that these results suggest that the process of apoptosis may be triggered in tissues which have recovered their normal energy state or which had no detectable average diffusion coefficient changes during hypoxia. These experiments suggest that \textsuperscript{99m}Tc-labeled annexin V imaging is useful to identify neonates suffering from acute hypoxic/ischemic injury. This diagnostic tool and, potentially, other diagnostic MR spectroscopy tools may lead to early administration of neuroprotective agents, if and when these neuroprotective agents are identified. The use of this technique in the future in children may lead to a better understanding of hypoxic/ischemic injury in these children.

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99mTc Annexin V Imaging of Neonatal Hypoxic Brain Injury
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