Evolution of Brain Injury After Transient Middle Cerebral Artery Occlusion in Neonatal Rats

Nikita Derugin, MA; Michael Wendland, PhD; Kanji Muramatsu, MD, PhD; Timothy P.L. Roberts, PhD; George Gregory, MD; Donna M. Ferriero, MD; Zinaida S. Vexler, PhD

Background and Purpose—Stroke in preterm and term babies is common and results in significant morbidity. The vulnerability and pathophysiological mechanisms of neonatal cerebral ischemia-reperfusion may differ from those in the mature cerebral nervous system because of the immaturity of many receptor systems and differences in metabolism in neonatal brain. This study details the neuropathological sequelae of reperfusion-induced brain injury after transient middle cerebral artery (MCA) occlusion in the postnatal day 7 (P7) rat.

Methods—P7 rats were subjected to 3 hours of MCA occlusion followed by reperfusion or sham surgery. Diffusion-weighted MRI was performed during MCA occlusion, and maps of the apparent diffusion coefficient (ADC) were constructed. Contrast-enhanced MRI was performed in a subset of animals before and 20 minutes after reperfusion. Triphenyltetrazolium chloride (TTC) staining of the brain was performed 24 hours after reperfusion. Immunohistochemistry to identify astrocytes (glial fibrillary acidic protein), reactive microglia (ED-1), and neurons (microtubule-associated protein 2) and cresyl violet staining were done 4, 8, 24, and 72 hours after reperfusion.

Results—On contrast-enhanced MRI, nearly complete disruption of cerebral blood flow was evident in the vascular territory of the MCA during occlusion. Partial restoration of blood flow occurred after removal of the suture. A significant decrease of the ADC, indicative of early cytotoxic edema, occurred in anatomic regions with a disrupted blood supply. The decline in ADC was associated with TTC- and cresyl violet–determined brain injury in these regions 24 hours later. The ischemic core was rapidly infiltrated with reactive microglia and was surrounded by reactive astroglia.

Conclusions—In P7 rats, transient MCA occlusion causes acute cytotoxic edema and severe unilateral brain injury. The presence of a prominent inflammatory response suggests that both the ischemic episode and the reperfusion contribute to the neuropathological outcome. (Stroke. 2000;31:1752-1761.)

Key Words: infant ■ middle cerebral artery occlusion ■ MRI, diffusion-weighted ■ reperfusion ■ stroke ■ rat

Stroke is commonly thought to be a disease of the elderly. However, stroke occurs commonly in children, although its incidence is frequently underestimated. Neonatal stroke often leads to neurological dysfunction, cerebral palsy, and epilepsy later in life. Of the strokes occurring in infants, 55% are of ischemic origin.1 Cerebrovascular accidents occur in 20% to 30% of infants born at <35 weeks of gestation, which is a much higher frequency than occurs in term babies or people older than 65 years.2 Neonatal stroke often involves the middle cerebral artery (MCA) territory in term infants.3

Previous studies determined that the response of the immature central nervous system (CNS) to hypoxia and ischemia differs considerably from that of the mature CNS. Notably, overexpression of CuZn-superoxide dismutase (CuZn-SOD) is associated with exacerbation of hypoxic-ischemic (HI) injury in immature brain,4 while CuZn-SOD overexpression is associated with reduced brain injury after ischemia in adult mice.5 Glutathione peroxidase activity is diminished after H-I in immature brain,6 but glutathione peroxidase is increased in adult brain after transient cerebral ischemia.7 Hypoglycemia produces opposite effects on the outcome of ischemia in neonatal and adult rats.2 After ischemia, apoptosis is associated with activation of caspase-3 and caspase-1 in the adult brain, while in neonates H-I activates only caspase-3.8 In addition, blockade of N-methyl-D-aspartate receptors causes pronounced apoptotic neurodegeneration in the immature brain9 but not in the adult brain.

Many factors may account for the different age-dependent patterns of injury. These include dynamic changes in regional distribution and regulation of cerebral blood flow (CBF) that occur during the first 2 weeks of life,2 incomplete myelination and immaturity of the blood-brain barrier,10 differences in...
Mechanisms of H-I have been studied in postnatal day 7 (P7) rodents with a combination of unilateral carotid artery ligation and 2 to 2.5 hours of systemic hypoxia.16,17 Animals of this age have brain development similar to that of near-term human babies.18 The combination of these 2 insults is required because unilateral carotid artery ligation or nonlethal hypoxia alone fails to produce a consistent pattern of histologically defined injury. Hypoxia plus ischemia, however, produces unilateral injury ipsilateral to the carotid artery ligation.2,11,19,20 Because both a hypoxic episode and permanent common carotid artery (CCA) ligation are required to produce CNS injury, this model does not mimic the pathophysiology of transient occlusion of CBF seen in human neonates.3

Reperfusion/reoxygenation after an ischemic episode can significantly exacerbate injury to brains of adults, as was demonstrated with the use of rodent models of focal permanent and transient cerebral ischemia (see References 21 and 22 for review). The limited antioxidative capacity of the immature CNS5,19 renders the brains of neonates even more susceptible to reoxygenation and oxidative stress than the brains of adults. To determine the role of reperfusion on the injury occurring in immature rat brains after focal cerebral ischemia, several models have recently been developed. A model of transient MCA occlusion was first developed in P14-P18 spontaneously hypertensive rats.23 We subsequently modified this model to accommodate P7 rats.24 The ischemia produced by transient occlusion of the MCA with a suture causes infarction in the cortex and caudate nucleus 24 hours after reperfusion.23,24 Another model has recently been described in P7 rats that employs a combination of permanent MCA occlusion and transient CCA occlusion.25 In this model injury primarily affects the cortex, and the model is associated with a characteristic pattern of apoptosis that occurs 48 hours after reperfusion.25

The present study was designed (1) to determine that our model of transient MCA occlusion in P7 rats produces both severe reduction in blood supply after suture occlusion and reperfusion of the brain after suture removal and (2) to characterize acute neuropathological changes after transient MCA occlusion. By employing 2 MRI techniques, we determined that transient MCA occlusion nearly completely disrupted the blood supply and caused early cytotoxic edema of the brain regions perfused by the MCA. We also determined that transient MCA occlusion results in severe injury, accumulation of reactive microglia, and elaboration of focal astrogliosis.

Materials and Methods

Animals

All animal research was approved by the University of California at San Francisco Committee on Animal Research and was performed in accordance with the Guide for the Care and Use of Laboratory Animals (US Department of Health and Human Services, Publication No. 85-23, 1985). Ten female Sprague-Dawley rats with a 6-day-old litter (9 to 10 pups per litter) were obtained from Simonson Labs (Gilroy, Calif). The mother and her pups were given food and water ad libitum and housed in a temperature/light-controlled animal care facility until the pups were 7 days old.

P7 rat pups (weight, 14.7 ± 1.5 g) were subjected to transient MCA occlusion, as previously described.24 Briefly, each pup was anesthetized with 1.5% to 3% isoflurane in a mixture of 70% N2O and 30% O2. Body core temperature was maintained between 35.5°C and 37°C with the use of an overhead radiant heat source and a circulating water jacket. All procedures were performed in spontaneously breathing pups. The CCA was exposed through a midline cervical incision. A precoated24 suture (7-0; Ethicon) was inserted through the external carotid artery (ECA) into the internal carotid artery (ICA) and advanced 8.5 mm past the ECA-ICA bifurcation to occlude the MCA. In sham-operated rat pups, the suture was advanced 3.5 mm past the ECA-ICA bifurcation. The skin was closed with 3 interrupted sutures. After recovery from anesthesia, the pups were returned to their dams. Reperfusion of the brain was achieved by removing the suture 3 hours after MCA occlusion. Rat pups were killed 30 minutes or 4, 8, 24, or 72 hours after reperfusion. The suture was not removed in 4 rats to produce permanent 24-hour MCA occlusion.

A total of 72 rats were subjected to MCA occlusion. Six rats were excluded from the study: 3 rats bled during reperfusion, 2 appeared to hemorrhage when they were killed, and 1 died during the MRI session. Of the 66 rats studied after MCA occlusion, 15 rats were killed after 24 hours of reperfusion to determine the patterns of injury using triphenyltetrazolium chloride (TTC) staining. Sixteen rat pups were used to test whether diffusion-weighted (DW) MRI during MCA occlusion can be used as an indicator of ultimate infarction at 24 hours after reperfusion. Then DW MRI was used as a criterion of injury in the remaining 35 animals. Of the 51 rats that underwent DW MRI, 23 had injury. The brains of rats with extensive injury identified by DW MRI were perfusion-fixed and used for immunohistochemistry 4 (n = 4), 8 (n = 4), 24 (n = 6), or 72 (n = 5) hours after reperfusion or 24 hours after permanent MCA occlusion (n = 4). Five of 51 rat pups, in addition to DW MRI, underwent contrast-enhanced MRI immediately before and 20 minutes after reperfusion. For DW MRI, the rats were reanesthetized 2 to 2.5 hours after MCA occlusion. Rat pups undergoing DW and contrast-enhanced MRI were reanesthetized 2.5 hours after MCA occlusion, and their left jugular vein was cannulated with stretched polyethylene (PE10) tubing for intra-venous contrast agent administration. For MRI measurements, spontaneously breathing animals (1.2% isoflurane plus 70% N2O and 30% O2) were placed in a polypropylene tube and inserted into the imaging coil. An external heat source blew warm air around the animal to maintain the body temperature at a normal level during each MRI experiment. Rectal temperatures were measured before and after each MRI experiment.

Magnetic Resonance Imaging

MRI was performed with a Bruker Omega CSI (2T) system (Bruker Medical Systems, Inc) equipped with Acustar S-150 self shielded gradients (20 G/cm) with the use of a 24-mm ID home-built birdcage radiofrequency coil. Instrument settings for DW spin-echo, echo-planar images were as follows: field of view, 35 mm; data matrix, 128×128 (resolution, 0.27 mm per pixel); repetition time, 4000 ms; echo time, 60 ms; slice thickness, 2 mm; diffusion field gradient pulse duration, 15 ms; gradient waveform, half sine wave; time separation for gradient pulses, 31.7 ms; spectral width, 200 KHz; and data acquisition time, 82 ms. To calculate the apparent diffusion coefficient (ADC), a set of images was obtained in which the amplitude of the diffusion-sensitizing gradient pulses was increased from 0 to 10 G/cm,26-28 in which the b factor increased from 0 to 1800 s/mm2. The ADC was calculated with the following equation: S(b)/S0 exp(−b ADC), where S(b) refers to the pixel signal intensity as a function of the b factor, and S0 refers to signal intensity of the pixel when the diffusion gradient amplitude is zero.
DW images were acquired in the coronal plane at the level of the anterior commissure after MCA occlusion or sham surgery. A single heavily DW image (7 G/cm) was obtained first. Rat pups with apparent unilateral hyperintensity on these images then had a set of 11 coronal echo-planar DW images obtained while the b values increased from 0 to 1800, as we previously described. ADC maps were obtained. The ADC measurements were performed in regions of interest (ROIs) that included the MCA vascular territory, both the cortex and caudate nucleus, in the hemisphere ipsilateral to the occlusion and in matching anatomic regions on the contralateral side. The size of the anatomic region with diminished ADC values was measured and expressed as a percentage of the hemisphere ipsilateral to the occlusion. If no evidence of hyperintensity on the occluded side of the brain was apparent on heavily DW image in the plane of the anterior commissure, no DW images with different b values were acquired. However, an additional single DW image was acquired ~2 mm posterior to the anterior commissure to determine the presence of a lesion in the posterior caudate nucleus and to compare this lesion with the lesion apparent on TTC staining.

Perfusion-sensitive MRI was performed with a combination of echo-planar, spin-echo MRI and a bolus of magnetic susceptibility contrast agent, 0.37 mmol/kg spironolactone (Nycoden Amersham AS), as previously described. The presence of the agent was detected as a transient signal loss in tissue surrounding the perfused microvasculature and converted in a $\Delta R_2^*$ format defined by the equation $\Delta R_2^* = -\log(S(t)/S(0))/TE$, where $S(t)$ and $S(0)$ are signal intensities at time t and time 0, respectively, and TE is echo time. Since the contrast agent effect, characterized by $\Delta R_2^*$, was shown to be approximately proportional to its concentration, estimates of relative cerebral volume can be obtained by temporal integration. $\Delta R_2^*$ values were measured in the ROIs as described for the ADC measurements.

**Histology and Immunohistochemistry**

For TTC staining 24 hours after reperfusion, rat pups were anesthetized with sodium pentobarbital (Nembutal, Abbott Labs), decapitated, and the brains were removed and cut into 3 to 4 coronal sections. TTC staining was accomplished by incubating coronal brain sections in a 2% TTC-saline solution for 10 minutes at 37°C. Twenty-four hours later, TTC staining patterns were recorded on a flat-bed color digitizer connected to a Macintosh computer. The size of moderate to complete TTC discoloration was determined in serial sections by computer-assisted image analysis (NIH Image Software) and expressed as a percentage of the hemisphere. In addition, the size of region with decreased ADC value and the size of region with TTC discoloration on a single coronal section that most closely resembled the one obtained during DW MRI were correlated after extensive (n=6) and no (n=5) injury. For cresyl violet staining and immunohistochemistry, rats were perfused with ice-cold 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4) through the heart. The brains were removed and left for postfixation for 24 hours. The brains then were transferred to a 30% sucrose/PBS solution and left at 4°C for 72 hours. Brains were flash-frozen with the use of an isobutanol/dry ice mixture, stored at −70°C, and cut into 15-μm coronal sections with a cryostat. After quenching with 1% H2O2 and blocking nonspecific staining in 100 mmol/L Tris-buffered saline (TBS, pH 7.4) that contained 10% goat serum for 30 minutes, we incubated the sections for 1 hour with monoclonal anti–glial fibrillary acidic protein (GFAP) antibody (1:50; ICN); ED-1 antibody, a monoclonal antibody specific for reactive microglia and macrophages (1:100; Serotec); or monoclonal anti–microtubule-associated protein 2 (MAP2) antibody (1:250, Sigma) in 100 mmol/L TBS containing 2% goat serum, 0.1% Triton X-100, and 0.1% BSA (TX/BSA). After three 10-minute washes in TBS/TX/BSA, the sections were incubated for 1 hour with appropriate biotinylated goat anti-rat or anti-mouse secondary antibody (1:200, Vector Labs). After three 10-minute washes in TBS/TX/BSA, sections were incubated with ABC reagent (Vector Labs) for 1 hour, and, after three 10-minute washes in TBS, the sections were incubated with diamobenzidine and 0.05% H2O2 for 1 to 5 minutes. Sections were washed 3 times for 10 minutes in TBS. Some of the sections were counterstained with cresyl violet. The sections were then dehydrated and mounted on slides. Representative sections were scanned (Alpha Imaging Software) and analyzed.

ED-1 immunoreactivity was semiquantified in the ischemic core and penumbra on a scale of 0 to 4: 0, no or few ED-1 immunoreactive cells; 1, random immunoreactive cells; 2, a discrete region of immunoreactive cells; 3, many strong immunoreactive cells; and 4, the entire region is infiltrated with immunoreactive cells. ED-1 immunoreactive cells were always observed bilaterally in the corpus callosum and periventricular regions and were not scored.

Data are shown as mean±SD. Statistical analysis was accomplished with the use of factorial 2-way ANOVA and post hoc significance testing with Bonferroni unpaired test (Statview V, Abacus Software). A P value of <0.05 was considered significant.

**Results**

The size and distribution of injury were determined by TTC staining 24 hours after reperfusion. The brains of sham-operated rat pups were bilaterally stained. Unilateral moderate to complete TTC discoloration was observed in 20 of 31 rats. In 15 of these 31 rats, TTC discoloration was observed in the MCA vascular distribution (ie, cortex and subcortical areas) (Figure 1). Discolored areas included 34.7±9.3% of the hemisphere ipsilateral to the occlusion. In 5 rat pups, only a discrete region of discoloration was present in the posterior caudate nucleus in the hemisphere ipsilateral to the occlusion. Many factors can account for occurrence of a more discrete region or no lesion at all in some of the animals. As we noted earlier, suture coating, 3-dimensional proximity of the suture tip, and the distance the suture was advanced may substantially affect induction of a reproducible infarction. If entrance to the MCA is physically blocked by the suture, but the suture tip does not occupy the entire cross section of the ICA, the presence of some blood flow from the ICA may be sufficient to protect the entire MCA territory, thus causing no
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...injury or injury only to the caudate nucleus. This notion is consistent with the recent observation that electrocoagulation of either the MCA or CCA in P7 rats is insufficient to produce CNS damage. Only a combination of permanent MCA coagulation and a transient (1 hour) occlusion of the CCA consistently produced unilateral cortical injury.

To determine whether slowed water diffusion in the vascular territory of the occluded MCA can be used as a noninvasive early indicator of ultimate infarction, DW MRI was performed during the occlusion and was followed by TTC stain 24 hours after reperfusion in the same animals (n=16). In 6 rat pups, hyperintensity on the DW image and the decreased ADC was observed in 66±2% of the hemisphere ipsilateral to the MCA occlusion in a single chosen plane (Figure 1A), and TTC discoloration was detected in closely corresponding brain regions 24 hours later (Figure 1B). In these regions, the ADC was 0.47×10⁻³±0.12×10⁻³ cm²/s, significantly lower than the value of the matching region on the side contralateral to the occlusion, which was 0.96×10⁻³±0.10×10⁻³ cm²/s (P<0.001). In 4 rats, when DW hyperintensity was observed only in a discrete region having the shape and location of the posterior caudate nuclei, TTC discoloration was restricted to a small area of the posterior caudate. In 6 rats that failed to exhibit DW hyperintensity in any plane studied, no TTC discoloration was observed 24 hours later. The size of region with decreased ADC value and the size of region with partial to complete TTC discoloration on a single coronal section that most closely resembled the one obtained during MRI matched closely (r=0.956). Since information from DW MRI enabled us to predict histopathological outcome, TTC histology was omitted for the rest of the study. The presence of decreased ADC within the cortex and caudate nucleus after MCA occlusion was used as a criterion for defining successfully occluded vessels.

The extent of disruption of the cerebral microcirculation after MCA occlusion and suture removal was determined by performing contrast-enhanced MRI in rats with a demonstrated ADC decrease on the side ipsilateral to the occlusion. In all animals studied, severe unilateral perfusion deficits were observed 3 hours after MCA occlusion. The studies were done 3 hours after MCA occlusion (A) or 20 minutes after reperfusion (B). C and D, Time courses of ΔR²* derived from images shown in A and B, respectively. A sharp peak of ΔR²* over time is observed in the contralateral, but not in the ipsilateral, MCA vascular territory during the occlusion (C). After reperfusion, there is a transient increase in ΔR²* observed in the region not previously perfused on the side ipsilateral to the occlusion (D).

Evolution of brain injury was determined with the use of cresyl violet staining (Figure 3). Cell morphology and cytoarchitecture of the cortex were normal in the hemisphere contralateral to ischemia (Figure 3A, 3E, 3G, 3I, and 3K). In frontoparietal and frontotemporal cortex ipsilateral to MCA occlusion, individual shrunken neurons were evident 4 hours after reperfusion (Figure 3B). The injured region continued to expand over time, and patches of shrunken neurons (Figure 3C and 3D) were observed 8 hours after reperfusion. By 24 hours, severe unilateral injury was obvious on both cresyl...
violet (Figure 3F and 3H versus 3E and 3G) and TTC (Figure 1B) stain in the frontoparietal and frontotemporal cortex ipsilateral to the MCA occlusion. There were no obvious signs of injury in the cingulate cortex (Figure 3F). Neuronal loss and disorganization of the infarcted region, as defined by cresyl violet staining, was paralleled by a complete loss of MAP2 immunoreactivity in this region 24 hours after reperfusion (Figure 4B versus 4A). The intensity of MAP2 immunostaining remained unchanged in matching regions contralateral to ischemia (Figure 4A and 4B) as well as in the cingulate cortex ipsilateral to ischemia (Figure 4C). Seventy-two hours after transient MCA occlusion, the extent of injury, as defined by cresyl violet staining, progressed within the core (Figure 3J). No obvious signs of injury were evident in the cortex of the opposite hemisphere (Figure 3K). Compared with transient MCA occlusion, damage within the frontoparietal and frontotemporal cortex ipsilateral to MCA occlusion was greater 24 hours after permanent MCA occlusion (Figure 3L).

Transient MCA occlusion in P7 rats evoked an acute inflammatory response. Reactive astrocytes, ie, GFAP immunoreactive cells, were seen bilaterally in the corpus callosum of sham-operated animals (not shown). Few GFAP immuno-

Figure 3. Evolution of brain injury after MCA occlusion in a P7 rat pup. Cresyl violet staining was performed after 3 hours of MCA occlusion and 4 hours (A and B), 8 hours (C and D), 24 hours (E through H), and 72 hours (I and J) of reperfusion or 24 hours after permanent MCA occlusion (K and L). Intact morphology of the neurons is seen in the hemisphere contralateral to MCA occlusion (A, E, G, and I), while the spread of injury over time is obvious in the hemisphere that is ipsilateral to MCA occlusion (B through D, F, H, and J). Arrows point to individual shrunken neurons in B or groups of shrunken neurons in C and D. More severe injury occurred after permanent than after transient MCA occlusion. Magnification ×4 in C, E, G, and I; ×20 in A, B, D, and G through I.

Figure 4. Neuronal loss and astrogliosis after transient MCA occlusion in a P7 rat pup 24 hours after transient MCA occlusion. A through C, MAP2 immunoreactivity in the hemisphere contralateral (A) and ipsilateral to the occlusion (B and C). Neuronal architecture is intact in the hemisphere contralateral to MCA occlusion (A) and in the penumbra (arrows, B), while no immunoreactive cells are observed in the ischemic core (B). Arrows in C demarcate the edge of the loss of MAP2 immunoreactivity. D through F, GFAP immunoreactive cells are seen bilaterally in the white matter tracts (D and E). Prominent GFAP immunoreactivity is seen in the penumbra (E and F) but not in the ischemic core. Arrows in F point to reactive astrocytes. Magnification ×4 in A, B, D, and E; ×40 in C and F.
Discussion

In this study we characterized the evolution of ischemic injury occurring acutely after reperfusion. Transient suture occlusion of the MCA of neonatal P7 rats resulted in major disruption of the blood supply within the MCA vascular territory, and removal of the suture resulted in re-establishment of the blood supply, as shown by MRI-based determination of cerebral microcirculation. Cytotoxic edema in the ischemic region occurred shortly after MCA occlusion and was followed by severe injury in the same region 24 hours after reperfusion. The ischemic core was rapidly infiltrated with reactive microglia and surrounded by reactive astroglia, supporting the notion that both the ischemic episode and the reperfusion phase contribute to the injury.

We used DW imaging and ADC values as a noninvasive measure of early posts ischemic disturbances of brain homeostasis and showed that an early decrease in the ADC value reliably predicts ischemic injury in neonatal animals 24 hours after reperfusion. A decrease in the ADC was previously observed by us and by others in adult animals during the early stages of cerebral ischemia, after N-methyl-d-aspartate injection, during spreading depression, and in neonatal rats soon after global ischemia and H-I. DW image intensity depends on the ADC of the water molecules. By assuming that there is a fast water exchange between the extracellular and intracellular spaces, it has been postulated that the measured ADC is a weighted average of both fractions. Reduced blood flow and the associated decrease in high-energy phosphates, Na+/K+-ATPase activity, and the limited ability of cells to maintain their membrane potential increase intracellular Na+ and the net movement of osmotically driven water from the extracellular to the intracellular space. This results in cytotoxic edema. Water flux from the relatively fast diffusion in the extracellular space to the intracellular compartment, where diffusion is slowed, results in the net slowing of water diffusion. It was also suggested that changes in water diffusivity in each of the extracellular and intracellular compartments contribute to a decrease in ADC after H-I. The presence of net slowing of water diffusion associated with cytotoxic edema can be detected early after ischemia by generating spatial maps of the ADC and by analyzing the ROI of these spatial maps in the ischemic or partially perfused regions and in the corresponding contralateral anatomic areas.

Our data show that a significant decrease of the ADC values 3 hours after MCA occlusion was always followed by TTC- and Nissl-documented injury and that regions with decreased ADC matched the regions of infarction present 24 hours after reperfusion. Furthermore, the fact that no TTC- or
cresyl violet–defined injury was observed at 24 hours in brain regions where signal intensities on DW images were normal strongly suggests that the significant decrease of the ADC early after MCA occlusion reliably predicts subsequent severe ischemic brain injury in the neonatal rat. The ADC values in the hemisphere contralateral to the occlusion are higher in this study than values that we and others observed in the brains of adult animals.27,28 This observation is thought to be related to a decrease of the ADC values in intact brains with increasing age because the number of compartments with slower water diffusivity increases during brain myelination.35 Our observation is consistent with previous observations for rats of this age.35,36 The ADC values in the occluded MCA territory, however, are similar in P7 rats and in adult rats, perhaps representing a maximum of water redistribution between the intracellular and extracellular spaces.

Since the regions with abnormal ADC matched the regions of ultimate infarction 24 hours after reperfusion, we used decreased ADC as a criterion for delineating the “ischemic core” and defined the neighboring regions without a significant decrease in ADC to be the “penumbra.” Ischemia-reperfusion was associated with substantial progressive neuronal loss and with shrinkage of many of the remaining neurons in the ischemic core during 24 hours after reperfusion but not in the penumbra. Few shrunken or missing neurons were apparent in the region of the developing infarction 1 hour after reperfusion. Loss of neurons and increased number of shrunken neurons continued to progress over time within the same anatomic region. Twenty-four hours after reperfusion, there was a clear line of demarcation between the severely injured region supplied by the MCA and the frontoparietal and cingulate cortical regions with the preserved neuronal architecture and network (Figure 3). Complete loss of MAP2 immunoreactivity was observed in the ischemic core 24 hours after reperfusion (Figure 3E). MAP2 immunoreactivity in the ipsilateral region adjacent to the ischemic core was similar to that in the contralateral hemisphere. Absence of MAP2 immunoreactivity in severely injured tissue is thought to occur because MAP2 is cleaved in dying neurons.40

A nearly complete focal obstruction of the cerebral microcirculation for 3 hours, followed by recirculation, was sufficient to evoke reactive astrogliosis in our model. GFAP immunoreactive cells were aggregated in the cortical regions outside the ischemic core and in a lesser extent in the caudate nucleus, as well as within and around the corpus callosum. GFAP immunoreactivity in the hemisphere contralateral to the ischemia was predominantly seen around the corpus callosum and was comparable to that in sham-operated rats. Our findings support the notion that the magnitude of reactive astrogliosis in the neonatal brain depends on both the nature and the severity of the injurious stimuli. In P7 mice, application of traumatic stimuli alone did not evoke an astrogliotic response.41 However, profound gliosis occurred after prolonged use of traumatic stimuli or after acute trauma combined with the application of cytokines. Severe focal ischemic injury to the neonatal brain in this and other studies42 and after H-136,43 is associated with a rapid induction of reactive astrogliosis and subsequent scar formation in regions that surround the injury.36,42,43

Accumulation of microglia has been reported to occur in the brains of neonatal P7 rodents after H-I.44,45 The presence of reactive microglia, as identified by ED-1 immunoreactivity, occurred by 24 hours after transient MCA occlusion in our animals and after permanent MCA occlusion plus transient CCA occlusion.42 Interestingly, microglia activation occurred more rapidly after both H-I and focal ischemia in the brains of P7 rats than in the brains of adult rats after ischemia-reperfusion. However, patterns of regional distribution of the microglial response after H-I and focal transient ischemia were different in P7 rats. A marked increase in the number of ED-1 immunoreactive cells was observed 24 hours after the H-I insult, predominantly in the hippocampus ipsilateral to the occluded CCA.45 When activation of microglia was determined by immunoreactivity to major histocompatibility complex II (OX-18), the appearance of activated microglia was delayed but was apparent in the hippocampus, cortex, and thalamus 4 to 14 days after the H-I. Increased OX-18 immunoreactivity correlated well with the loss of MAP2 immunoreactivity in the same regions.44 In our model, ED-1 immunoreactive cells were visible within the forming infarction 8 hours after reperfusion. Twenty-four hours after reperfusion, the ischemic core was infiltrated with reactive microglia. No such cells were present in the penumbra regions and on the unlesioned side of the brain. The magnitude of the microglial response was further increased 72 hours after reperfusion, a duration of reperfusion that is reportedly associated with the accumulation of ED-1–positive cells in adult rodent models of transient MCA occlusion. Blood-derived macrophages and activated resident microglia may constitute 2 distinct phagocytic cell populations observed days after reperfusion. Both of them are ED-1 immunopositive, exert similar functions, and have similar morphology. In our experiments reactive microglia were seen at 72 hours not only in the ischemic core but also in the penumbra, indicating spread of the injury. The triggering role of reperfusion in the activation of microglia became apparent when we compared the patterns of ED-1 immunoreactive cells 24 hours after transient MCA occlusion and permanent MCA occlusion. After permanent MCA occlusion, few positive cells were seen within the ischemic core. ED-1–positive cells were observed mostly on the border between the infarcted tissue and tissue with minimal morphological signs of injury. The patterns of microglial response after transient and permanent disruption of the blood and oxygen supplies indicate a potentially important role of reperfusion in the mechanism of ischemia-induced injury to the neonatal brain. Differences in regional distribution of the microglial response after H-I and MCA occlusion support the notion that accumulation of activated microglia predominantly occurs in severely injured tissue.

The exact mechanisms of this activation and the role of microglia-mediated effects on the pathophysiology of neonatal stroke are yet to be unraveled. In general, reactive microglia may exert a bidirectional role after cerebral ischemia, a long-term beneficial effect due to involvement in removal of neuronal debris, and a short-term injurious effect
due to release of proinflammatory cytokines and reactive oxygen metabolites after reperfusion. It is not clear whether the more rapid activation of microglia in neonatal brain is associated with distinct developmental stage specific properties of microglia or whether it is due to a more robust inflammatory response or a larger magnitude of oxidative stress that is associated with reoxygenation. Cytokines interleukin (IL)-1, IL-6, and transforming growth factor-β, which are produced by activated microglia after an H-I insult, cause injury by affecting leukocyte-endothelial interactions, by initiating astrocyte hypertrophy and/or proliferation, and by activating microglial inducible nitric oxide synthase (iNOS). IL-1 receptor antagonists and inhibition of iNOS protect the neonatal brain against an H-I insult. The persistent microglial hyperactivation caused by injury early in life may have serious consequences later through secretion of a variety of factors, aberrant axonal growth, gliogenesis, and angiogenesis.

Evidence of the contribution of neutrophils and chemokines to an acute inflammatory response to ischemia-reoxygenation of the immature brain is emerging. Neutrophil activation was observed after both H-I and focal cerebral ischemia in P7 rats. Neutrophils were persistent in the vessels and in the parenchyma after focal ischemia, but extravasation of these cells was either transient or limited after H-I. A profound increase in myeloperoxidase activity, an indicator of neutrophil functional activity, however, paralleled the lack of neutrophil extravasation. Depletion of neutrophils before H-I appeared to be protective. Recent data show chemokine induction, upregulation of β2-integrins, and the presence of lymphocytes in the brains of P7 rats after H-I. The CNS is traditionally thought of as an immunologically privileged site since it lacks a lymphoid system and antigen-presenting cells. The intact blood-brain barrier almost completely blocks the influx of immunoglobulins and complement. Ischemic injury to the neural parenchyma leads to local production of proinflammatory cytokines and chemotactic molecules. This is followed by secondary changes in the adhesion properties of the surrounding vascular endothelium and a site-specific chemotaxis of circulating lymphocytes. Since the blood-brain barrier is not fully mature shortly after birth, the time course and magnitude of neutrophil- and lymphocyte-mediated effects may differ substantially in immature and mature brain after reperfusion/reoxygenation. The scope of these events and their long-term effects are yet to be determined.

Severe injury after transient focal cerebral ischemia at P7, in association with the inflammatory response reported here, suggests that the interplay of underlying pathophysiological events in this model may differ from those observed after H-I in P7 rats and after transient ischemia in the mature CNS. The contribution of the 2 paths of neuronal death, apoptosis and necrosis, to injury in the ischemic core and penumbra and the role of reperfusion in the pathophysiology of transient cerebral ischemia are yet to be determined in this model. Recent studies have demonstrated some similarities in the time course of ischemia-induced apoptotic cell death between P7 and adult rats. The magnitude of programmed cell death during development, however, is higher than in the adult brain, and the mechanisms of ischemia-induced apoptosis appear to differ between these 2 ages. The focal nature of the insult produced by our model provides an opportunity to examine the mechanisms underlying both types of cell death in the neonatal brain and to make direct comparisons between the pathophysiological mechanisms in neonatal and adult animals.

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References

The study by Derugin and colleagues reports patterns of reperfusion-induced brain injury after transient middle cerebral artery (MCA) occlusion by suture methods in neonatal (P7) rats. Rats were subjected to 3 hours of MCA occlusion followed by various reperfusion periods. Diffusion-weighted MRI (DWI) was performed while triphenyltetrazonium chloride (TTC) staining was conducted at 24 hours. Immunocytochemistry to identify astrocytes (GFAP), reactive microglia (ED-1), and neurons (MAP2), as well as cresyl violet staining, was undertaken at 4, 8, and 24 hours. A significant decrease in apparent diffusion coefficient, indicative of early cytotoxic edema, was associated with areas of histopathological damage. Interesting data regarding the evolution of injury during reperfusion periods and the potential role of inflammatory processes in progressive injury are provided.

The investigators are to be congratulated on conducting such a difficult study in neonatal rats. The model should provide an important tool for investigation of the pathomechanisms underlying cell death in the neonatal brain. In this study, a significant decrease in apparent diffusion coefficient (ADC) values 3 hours after MCA occlusion was correlated with regions of infarction at 24 hours. The ability to predict patterns of ultimate histopathological damage using maps of ADC MRI at early postischemic periods is an important aim and one that is supported by the present data.

The authors also describe the temporal and regional distribution of reactive microglia, as identified by ED-1 immunoreactivity, and implicate microglia accumulation in reperfusion-induced damage. Although the importance of inflammatory processes in the spread of injury after cerebral ischemia is an active area of research, the presence or absence of inflammatory cells in a given region does not indicate cause-and-effect relationships. Future pharmacological studies that specifically target microglial activation will be needed to critically assess the importance of the microglia in ischemia-induced injury to the neonatal brain. Nevertheless, the well-described patterns of microglial activation after transient and permanent MCA occlusion suggest an important role of reperfusion injury in this model of focal ischemia.

A potential shortcoming of this study was that animals were allowed to live for only 24 hours prior to evaluation with TTC. Because recent studies have indicated that longer survival periods may be necessary to define ultimate histopathological outcome in ischemia and trauma models, it will be important in future studies to correlate early DWI and ADC values with patterns of histopathological damage in rats surviving many days after ischemia.

W. Dalton Dietrich, PhD, Guest Editor
Department of Neurological Surgery
University of Miami School of Medicine
Miami, Florida
Evolution of Brain Injury After Transient Middle Cerebral Artery Occlusion in Neonatal Rats
Nikita Derugin, Michael Wendland, Kanji Muramatsu, Timothy P. L. Roberts, George Gregory, Donna M. Ferriero and Zinaida S. Vexler

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