MRI Evaluation and Functional Assessment of Brain Injury After Hypoxic Ischemia in Neonatal Mice

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Background and Purpose—Severe perinatal asphyxia is an important cause of brain injury in the newborn infant. We examined early events after hypoxic ischemia (HI) in the 7-day-old mouse brain by MRI and related them to long-term functional effects and histopathology in the same animals at 4 to 5 weeks of age.

Methods—HI was induced in 7-day-old CD1 mice by exposure to 8% oxygen for 30 minutes after occlusion of the left common carotid artery. The resulting unilateral focal lesion was evaluated in vivo by MRI (T2 maps and apparent diffusion coefficient maps) at 3, 6, and 24 hours and 5 days after hypoxia. Locomotion and sensorimotor function were analyzed after 3 weeks. Four weeks after HI, the mice were killed, and cresyl violet–stained brain sections were examined morphologically.

Results—A decrease in apparent diffusion coefficient values in cortex on the affected side was found at 3 hours after HI. T2 values were significantly increased after 6 hours and remained so for 5 days. Maximal size of the lesion was attained at 3 to 6 hours after HI and declined thereafter. Animals with MRI-detected lesions had decreased forward locomotion, performed worse than controls in the beam-walking test, and showed a unilateral hypotrophy in the cresyl violet–stained brain sections 4 weeks later.

Conclusions—The temporal progression of the damage after HI in 7-day-old mice differs from that of the adult brain as judged by MRI. The early lesions detected by MRI were related to functional impairments for these mice in near-adult life. (Stroke. 2002;33:1405-1410.)

Key Words: cerebral ischemia ■ hypoxia ■ magnetic resonance imaging ■ newborn ■ mice

Cerebral hypoxic ischemia (HI) is an important cause of brain injury in the newborn infant, with a risk of neurological sequelae such as cerebral palsy, epilepsy, or death. It is therefore important to study the mechanisms and consequences of HI injury in the immature brain. Since several reports show that the immature brain reacts differently to hypoxia and ischemia than does the mature brain, it is important to use an experimental model of the appropriate age. The brains of rats and mice are thought to be as mature at 7 to 10 days as third trimester human fetuses with regard to parameters such as number of synapses, neurochemical development, and cortical organization.

The combination of ischemia by unilateral ligation of the common carotid artery and timed exposure to 8% oxygen results in focal cerebral HI in the 7-day-old rat. In this well-established model, cerebral blood flow is restituted immediately after the period of hypoxia, and hence the model incorporates both focal brain ischemia and subsequent reperfusion. This model has recently also been characterized in mice and is considered to share important features with birth asphyxia in the human neonate with regard to blood flow changes and cellular metabolic derangements. Because the model allows long-term survival, it is also possible to evaluate long-term consequences, such as functional impairment.

MRI methods have been used in experimental animals for early detection and delineation of the area at risk for ischemic injury in the adult brain. The aim of the present study was to combine results from MRI at early time points after neonatal HI with functional assessments after several weeks. In particular, we have (1) examined the temporal evolution of injury after HI in the immature mouse brain and (2) combined acute MRI with functional and histopathological evaluation of the same individuals in adulthood.

Materials and Methods

Surgical Procedure

All experiments involving the use of animals followed the European Community Regulations and were approved by the regional animal ethics committee. A total of 38 CD1 mice (Charles River, Saint
Aubin les Elbeuf, France) of both sexes were used for the experiments. Animals were maintained on a regular light/dark cycle (light on from 7 AM to 7 PM) with free access to food pellets and tap water.

The experimental rat model was modified to suit immature mice by limiting the time the animals are exposed to hypoxia. At postnatal day 7, mice weighing 4 to 5 g (n=28) were anesthetized with 1% isoflurane in air. The left common carotid artery was dissected out, freed from the left vagal nerve, and occluded by electric coagulation with the use of low power. Two animals were sham operated, ie, they were anesthetized, the incision was made, and the vagal nerve was dissected free, but the artery was not occluded. The incision was sutured, and total surgery time usually did not exceed 2 minutes. Body temperature was maintained at 35°C. Four animals that bled during the surgery were excluded from the study. After surgery, the pups were returned to their dams for 1 hour to recover from surgery and to feed. The pups were then placed in an incubator within a chamber, through which a humidified atmosphere of 8% O2 and 92% N2 was flushed during the 30-minute exposure to hypoxia. The chamber temperature was kept constant at 35°C. After exposure to hypoxia, the pups were kept in the incubator in air at 35°C for 30 minutes. During the hypoxia, 2 of the animals died. Those that survived were returned to their dams until it was time for MRI. Four mice died within hours after HI as a result of maternal cannibalism. It is possible that if each mouse had been imaged only once or if only a few pups in each litter had been included in the study, the dams would have been less stressed, and this might have reduced the late mortality. However, the disadvantages of such a strategy are obvious: more animals are needed, and it is impossible follow the same animal over time, which is the ultimate advantage of MRI techniques.

MRI Protocol
A group of 8 control animals that had not undergone surgery or HI and 16 mice that were subjected to HI were imaged 1, 2, or 3 times at the following time points: 3 hours, 6 hours, 24 hours, and 5 days after HI. In addition, 2 sham-operated mice were examined 24 hours after surgery; their MRI parameters did not differ from those in controls. Before MRI, the animals were anesthetized (isoflurane 1% to 1.5% in air), and rectal temperature was monitored continuously with a digital thermometer with a superfine probe (REI-3, BAT-10, Physitemp); body temperature was kept at 35°C with a stream of warm air in the magnet. The imaging protocol usually took 1.5 hours. The skull was immobilized by placing the mouse in a belly-down position in a custom-built whole body mouse holder. MRI analysis was performed on a Biospec Avance 47/40 spectrometer (Bruker, Karlsruhe) operating at 4.7 T with the use of a custom-built semicircular radiofrequency resonator with a 24-mm inner diameter. T2-weighted images (T2WI) were obtained with field of view 25 mm2, acquisition matrix 256×256, slice thickness 0.5 mm, repetition time 2.1 seconds, and 16 averages. The images were obtained with a multiple echo sequence with equally spaced echoes 6 ms apart. Diffusion-weighted images were obtained by using a pulsed field gradient spin-echo technique with an echo time of 15.1 ms and 5 b values for diffusion weights (b=0, 20 000, 80 000, 180 000, 345 000 s/cm2). Diffusion sensitization was perpendicular to the image plane. Parameter maps were obtained from 2 parameter nonlinear fits (least-squares procedure). Images were analyzed with Paravision software. Infarcted area was delineated with the use of the T2 parameter map in the 2 hemispheres (Figure 2). Regions of interest within the lesion and on the contralateral side were indicated manually, and the mean of evaluations done by 2 independent investigators was used. Tissue with values within 2 SDs of the mean value in the corresponding location in control animals was considered normal; tissue with values deviating >2 SDs above mean value was considered infarcted.

Brain damage was evident in 12 of the 16 animals subjected to HI, the other 4 mice that did not have any detectable brain damage on T2WI at 3 or 6 hours after HI were not imaged at later time points (but were included in the behavioral evaluation).

Behavioral Tests
Behavioral tests were performed 3 weeks after HI. Ten controls (not subjected to HI), 14 mice of both sexes that were subjected to HI and had a brain lesion, and 4 mice that were subjected to HI but did not have any detectable lesion (on T2WI at 3 or 6 hours after HI) were evaluated for functional deficits. MRI had been performed on 8 of 10 in the control group, 12 of 14 in the HI/lesion group, and all 4 in the HI/no lesion group.

Since we expected the morphological lesion to involve several regions, including sensorimotor cortex, hippocampus, and striatum, we first used the open field activity test to screen for abnormal locomotor behavior and for habituation disabilities. The beam-walking test was selected because it is assumed to be sensitive to
disabled sensorimotor coordination. Locomotor activity was measured with the use of a 350×350×350-mm box with 4 photocells in both horizontal directions. The breaking of the light beams was recorded as counts by a computer (Kungsbacka Mät & Reglerteknik) during 5-minute periods. Sensorimotor performance was evaluated with the use of a beam-walking task. The mouse was placed at the end of a wooden beam 7 mm wide and 670 mm in length suspended 420 mm above ground and traversed the distance 3 times. The number of slips for each of the 2 hind limbs was counted separately.

On the first experimental day the animals were allowed to perform the beam-walking task once, and directly afterward they were habituated to the boxes for 30 minutes. This habituation procedure was performed once in the morning (habituation 1) and once in the afternoon (habituation 2). On the second experimental day, the procedure was repeated a third time (habituation 3). Directly after this they were injected intraperitoneally with 1 mg/kg of SKF 82958 C (6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrobromide; RBI). This drug causes a reproducible locomotor-stimulating effect via dopamine D1 agonism.

Histopathology

One week after the behavioral evaluation, animals were killed, and the brains were dissected out and frozen on dry ice, sectioned (20 μm), and stained with cresyl violet. The histopathological damage was scored with the use of light microscopy (scores from 2 blinded investigators were averaged) on a 0- to 24-point scale total for each brain. Three animals that had brain damage were not included in the correlation between MRI evaluation and histopathology because the ear tags of these mice had fallen off.

Statistical Analysis

Results are given as mean±SEM. Lesion area was analyzed by 1-sample t test, and other data from MRI, locomotor activity, and beam walking were analyzed by ANOVA. Data from histopathological evaluation were not normally distributed and were analyzed by Kruskal-Wallis test and Spearman correlation. The procedures in the Graph Pad Prism program were used.

Results

MRI

In most of the mouse pups subjected to HI, a lesion could be observed by MRI at 3 hours (Figures 1, 2, and 3), but distribution and severity varied between the animals. The tissues affected corresponded to those showing clear morphological damage 3 weeks later (Figure 1). At 21 days after HI, the midline was clearly shifted over to the lesioned side, indicating loss of brain substance. Changes were much more clear-cut in the lesioned hemisphere than on the contralateral side at all time points studied.

The apparent diffusion coefficient (ADC) values in the corresponding region initially decreased below normal values (seen as hypointense regions) and then increased; these regions became very bright by 5 days (Figures 2 and 3). Whereas ADC values were decreased at 3 hours after the
Figure 4. The area of lesion (expressed as percentage of whole brain cross-sectional area and assessed from calculated T2 maps) in animals subjected to HI differed from controls at all time points studied. Note that in the immature mouse brain, the lesion volume reaches its maximum before 24 hours. There was a time-dependent decrease in size of the lesion \((P<0.05)\). *\(^{P}<0.05\), **\(^{P}<0.01\), ***\(^{P}<0.001\).

The maximal extent of lesion, as judged by T2WI, was attained at the first time points examined and then declined progressively (Figures 2 and 4). In 2 animals that were imaged 3 days after HI, there was a further reduction in area of lesion compared with the measurements made at 24 hours after HI (not shown), indicating that there is no secondary increase in lesion size 3 days after HI. A time-dependent decline in lesion area \((P<0.05)\), calculated on the basis of T2 values, was observed (Figure 4). Changes in T2 values are considered to reflect altered degrees of freedom in protons, ie, changes in protein structures. The gradual reduction of the lesion may therefore mirror the resolution of an edema.

**Histopathological Evaluation**

Our data show that the area of injury that was detectable on T2WI at 3 hours after HI corresponded to the area of histopathological damage in the same animal seen 4 weeks later (Figures 1 and 5A). Conversely, severe histopathological brain damage was never seen in the mice that had no signs of infarction on T2WI at 3 or 6 hours after HI. However, in 1 of 4 mice, there was a mild columnar infarction in parietal cortex, although no striatal or hippocampal injury was detected in cresyl violet-stained sections. There was a positive correlation between the area of lesion seen at 3 to 6 hours after HI and histopathological score 4 weeks after HI \((r=0.8, P<0.01)\).

Interestingly, the mice that were subjected to HI but did not exhibit any detectable brain injury on T2WI at 3 or 6 hours after HI had a small but nonsignificant increase in total number of hind limb slips. Additionally, the number of hind limb slips was unaltered in all groups between habituations 2 and 3 but was significantly reduced \((P<0.001)\) after administration of a D\(_1\) agonist. Furthermore, the magnitude of the deficit in the animals subjected to HI tended to decrease after D\(_1\) stimulation.

**Behavioral Evaluation**

In mice with an MRI-verified brain injury, horizontal activity was decreased in the first habituation 3 weeks after HI (Table 1). The same tendency was seen in habituations 2 and 3 and after stimulation of locomotor activity with the use of a D\(_1\) agonist (SKF 82958C; not shown). The 4 mice that were subjected to HI but did not show evidence of damage on MRI did not exhibit any change in horizontal activity. Mice with an MRI-verified injury after HI also showed an increased number of hind limb slips in habituation 1 in the beamwalking test. The same tendency was seen in all habituations (Table 1).

**Figure 5. A.** Histopathological score 4 weeks after HI in animals that underwent MRI. The mice that were subjected to HI and had an MR-verified brain injury \((\text{HI/MR}+)\) displayed a significantly higher injury score \((P<0.05)\) than mice that were subjected to HI but did not have any detectable acute injury on T2WI \((\text{HI/MR}−)\). B. There was a positive correlation between area of lesion on T2WI at 3 to 6 hours after HI and histopathological score at 4 weeks after HI \((r=0.8, P<0.01)\).

**TABLE 1. Behavioral Evaluation 3 Weeks After HI in Neonatal Mice**

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=10)</th>
<th>HI/No Lesion (n=14)</th>
<th>HI/Lesion (n=14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Locomotor activity</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Horizontal activity,</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>habituation 1</td>
<td>1753.7±86.8</td>
<td>1819.8±105.2</td>
<td>1458.4±88.0*</td>
</tr>
<tr>
<td>Beam walking</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total slips</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Habituation 1</td>
<td>11.0±2.7</td>
<td>20.8±4.4</td>
<td>30.7±5.5*</td>
</tr>
<tr>
<td>Habituation 2</td>
<td>5.8±1.7</td>
<td>8.8±3.4</td>
<td>13.2±3.2</td>
</tr>
<tr>
<td>Habituation 3</td>
<td>6.0±1.5</td>
<td>11.7±3.4</td>
<td>11.3±2.4</td>
</tr>
<tr>
<td>After stimulation</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D(_1) agonist</td>
<td>2.1±0.7</td>
<td>2.3±0.3</td>
<td>3.0±0.8</td>
</tr>
</tbody>
</table>

Controls were not subjected to HI. Among animals that were subjected to HI, a majority had an MR-verified brain lesion \((\text{HI/Lesion})\). Open field horizontal activity was measured as counts during 30 minutes, and total (left- and right-sided) hind limb slips were recorded in a beam-walking test.

*Significant difference vs controls \((P<0.05)\).
In this study of HI in immature mice, we show very early changes using MRI that appear to predict not only histopathological changes but also behavioral deficits several weeks later. The results are discussed in relation to earlier data suggesting major differences between the mature and immature brain and the possibility that there might be differences between rat and mouse.

In the immature human15 or rat16–18 (Table 2) brain, ADC values are higher than in the mature brain. The same appears to be true in mice19,20: the control ADC values in the present study and in other studies of the immature rat were higher than those reported in most earlier studies in the mature brain (Table 2). This is probably not only an artifact caused by differences in the technical setup. Instead, a likely explanation is related to the unmyelinated state of the immature brain with higher extracellular space volume.21

Another interesting age-dependent difference relates to the time to maximal damage as judged by MRI findings. Thus, we found that the maximal area of affected brain tissue in juvenile mice was reached at 3 to 6 hours after HI (the first time points studied), whereas the maximal extent of focal ischemic lesion in adult mice is usually attained later (Table 2). A possible reason for this difference might be that different models are used to induce focal ischemia used in immature (present study) and in mature mice. Given the essential similarity in findings obtained with the use of different experimental models, this is probably not the only explanation. The alternative is that the mechanisms underlying posts ischemic damage differ in important ways between young and old mice. For example, the immaturity of the blood-brain barrier22 may affect the evolution of the injury. There are also other important differences between animals of different ages, eg, changes in cerebral blood flow during the first weeks of life, different glucose utilization, and immaturity of enzymes and receptors that are important for cerebral response to injury (for review, see Reference 7). In some respects there are similarities between young and adult mice, however. The temporal progression of changes in ADC and in T2 values in the present study was similar to that observed in the mature mouse brain.20 Our results also indicate that there are similarities between immature rat and mouse. The control ADC values essentially agree with previous studies investigating MRI in the 7-day-old rat brain (Table 2). Additionally, the progression of changes in ADC values after HI in the present study agrees with those seen in the 7-day-old rat brain.16,17 In the immature rat model of HI, the ADC values decreased first at 0 to 1 hour and then again from 5 to 24 hours after HI.16 We did not examine time points earlier than 3 hours after HI. Therefore, we do not know whether there is a biphasic time course similar to that observed in the rat.16 Our study confirms and extends the results from the 7-day-old rat brain in previous reports16,18 showing that the early MRI changes indicate the region at risk. Nedelcu et al16 showed that early changes in ADC values in the area supplied by the ipsilateral common carotid artery are followed by a histopathological injury in that region 5 days after HI. The only previous MRI study after middle cerebral artery occlusion in neonatal rat18 showed that a decreased ADC early after middle cerebral artery occlusion predicted severe ischemic damage 24 hours later. This finding is extended in the present study, in which increased T2 values and decreased ADC values in the ipsilateral hemisphere at 3 to 6 hours after injury (in 12 of 16 animals) were followed by hippocampal, cortical, and striatal damage with necrosis and tissue loss 4 weeks later in the same animals.

In addition to morphological evaluation of the brain damage, we have assessed functional changes. There are few experimental reports on functional deficits after focal ischemia in rodents, and even fewer have used neonatal models (Table 2). Our results on 7-day-old mice agree with the reports23,24 that show impaired sensorimotor function at 3 to 9 weeks after HI in 7-day-old rats. In accordance with our previous results in adenosine A2A knockout mice,6 the present study indicates that the beam-walking test might be more sensitive to minor ischemic damage in the cerebral cortex than the locomotor activity test. Thus, in the present study, impairment in beam walking was found not only in animals with a clear lesion detected by MRI; animals that had no detectable lesion on T2WI (and were subjected to HI) tended to have slightly more hind limb slips than controls as well. It is interesting to note that these deficits were significant only the first time the animals walked the beam. This could suggest that the defects that occur without any major loss of nerve tissue can be at least partly corrected by training. The
fact that the differences between groups in this test were least in animals that received a dopamine receptor agonist also argues that general motor activity and motivational aspects are important.

In the locomotor activity measurements, only the animals with a T2WI-verified infarction displayed an altered locomotor activity compared with controls. Data on locomotor activity changes after brain ischemia in the literature are contradictory. One previous study in rats showed that the locomotor activity was initially increased and then decreased after transient forebrain ischemia, and another study reported decreased locomotor activity 24 hours after focal ischemia (middle cerebral artery occlusion) in mice. Thus, the effect of cerebral ischemia on locomotion may depend on the model and evaluation time point.

In summary, we describe the temporal development of injury in the immature mouse brain after HI. We conclude that the maximal extent of the lesion is attained earlier in the immature than in the mature mouse brain. Moreover, in the animals that were found to have early changes on T2WI, long-term behavioral changes were detected. It is therefore suggested that MRI is a good tool for evaluating brain damage due to HI in neonatal mice and might be used in further studies that use genetically modified animals and early interventions with potential therapeutic agents.

Acknowledgments

This study was supported by Trygg Hansas Forskningsstiftelse, Konung Gustav V och Drottning Victorias Stiftelse, Swedish Medical Research Council (project 2553), Bank of Sweden Tercentenary Foundation, Knut and Alice Wallenberg Foundation, European Commission (Fifth Framework), Linnéa and Josef Carlsson Foundation, General Maternity Hospital Foundation, and “Stiftelsen Samariten,” Sweden. We want to thank Ann Sheldon and Donna Ferriero for valuable advice on the mouse model, Hugo Laggercrantz for surgical equipment, and Tomas Klasson for help with the MRI protocol.

References

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Stroke. 2002;33:1405-1410
doi: 10.1161/01.STR.000014608.78503.DB
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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