Reversal of Early Diffusion-Weighted Magnetic Resonance Imaging Abnormalities Does Not Necessarily Reflect Tissue Salvage in Experimental Cerebral Ischemia

Thomas M. Ringer, MD; Tobias Neumann-Haefelin, MD; Raymond A. Sobel, MD; Michael E. Moseley, PhD; Midori A. Yenari, MD

Background and Purpose—Diffusion-weighted MRI (DWI) can detect early ischemic changes and is sometimes used as a surrogate neurological end point in clinical trials. Recent experimental stroke studies have shown that with brief periods of ischemia, some DWI lesions transiently reverse, only to recur later. This study examined the histological condition of the tissue during the period of DWI reversal.

Methods—Rats underwent 30 minutes of middle cerebral artery occlusion followed by reperfusion. DWI images were obtained during ischemia and 3 to 5 hours, 1 day, and 7 days later. MRI scans were compared with histology (5 hours, n=5; 7 days, n=5) with the use of neuronal (microtubule-associated protein 2 [MAP2]) and astrocytic (glial fibrillary acidic protein [GFAP]) markers and heat-shock protein 72 (HSP72).

Results—DWI abnormalities reversed 3 to 5 hours after ischemia onset but recurred at 1 day. Four animals showed complete reversal of the initial DWI hyperintensity, and 6 showed partial reversal. When the 5-hour DWI was completely normal, there was significant loss of MAP2 immunoreactivity, comprising approximately 30% of the initial DWI lesion. However, GFAP staining revealed morphologically normal astrocytes. HSP72 immunoreactivity at 5 hours was extensive and corresponded to the initial DWI lesion.

Conclusions—After brief ischemic periods, normalization of the DWI does not necessarily imply that the tissue is normal. Neurons already exhibit evidence of structural damage and stress. Normal GFAP staining suggests that other nonneuronal cell populations may partially compensate for altered fluid balances at the time of DWI reversal despite the presence of neuronal injury. These observations suggest that caution is warranted when relying solely on DWI for assessment of ischemic damage. (Stroke. 2001;32:2362-2369.)

Key Words: astrocytes ■ cerebral ischemia ■ heat-shock proteins ■ histology ■ immunohistochemistry ■ magnetic resonance imaging ■ stroke ■ rats

Diffusion-weighted MRI (DWI) has been used increasingly in recent years to determine the evolution of ischemic tissue injury1,2 or to evaluate potential treatments in patients with experimental stroke3–6 and acute stroke.7–9 Particularly useful is the capability of DWI to detect early changes in ischemic tissue within minutes after focal cerebral ischemia.10–13 The DWI hyperintensity is thought to reflect the reduction of free water diffusion in the ischemic tissue13,14 and is presumed to be related to intracellular water accumulation (cytotoxic edema) due to loss of membrane ionic gradients.15–17 However, the exact physiological basis of the DWI lesion is still not completely understood.18

In previous studies it was shown that the hyperintense DWI lesions during ischemia decreased after onset of reperfusion in models of relatively brief periods (up to 1 hour) of transient middle cerebral artery occlusion (MCAO).12,19–21 The reversal of these acute DWI abnormalities was only transient, and ischemic lesions became detectable again at later time points by MRI and histology.22–27 We recently showed that even if DWI lesions reversed in the early reperfusion period after 30 minutes of ischemia, they recurred by 1 day.28 However, detailed histological analysis of this reversed tissue has not been performed, and whether there is underlying pathology in the reversed tissue is not known. In the present study we investigated the histological condition of the brain during this period of DWI reversal using neuronal and glial markers, as well as the 72-kDa heat-shock protein (HSP72).

Materials and Methods

Stroke Model

All experimental protocols were approved by the Stanford University Administrative Panel on Laboratory Animal Care. Ten male
Sprague-Dawley rats (Charles River, Wilmington, Del), weighing 300 to 340 g, were anesthetized by face mask with 3% halothane plus oxygen and air supplied in a ratio of 3:7. Halothane concentration was reduced to 1.5% during the surgery and further decreased to 0.75% to 1% during scanning. Rectal temperature was maintained at 37°C with a warm air circulation system. Pulse oximetry was used to monitor oxygen saturation and heart rate. An ischemic lesion was created within the territory of the middle cerebral artery (MCA) by introducing a 4-0 silicone-coated suture (Ethicon) into the distal common carotid artery. The suture was advanced into the internal carotid artery approximately 20 mm from the carotid bifurcation. Immediately after the occlusion, the head was fixed in a plastic headholder with ear bars and positioned in the MRI scanner. After 30 minutes of transient focal ischemia, the rats were removed from the magnet, the suture was withdrawn, and reperfusion was initiated. After 3 hours, 5 hours, 1 day, or 7 days following ischemia onset, the animals were reanesthetized and additional MRI scans were performed.

This study consisted of 2 groups. In 1 group (n=5) MRI was performed during ischemia (before reperfusion) and 5 hours after ischemia onset. These rats were killed immediately after the second MR scan by an overdose of halothane and transcardially perfused with normal saline. The brains were rapidly removed, sectioned into four 4-mm-thick blocks in the coronal plane, placed in embedding medium, and rapidly frozen on dry ice. MRIs were performed in the same manner during ischemia in the second group (n=5), but additional scans were obtained at 3 hours, 1 day, and 7 days after occlusion. After 7 days, the rats were killed and perfused with normal saline, followed by 4% paraformaldehyde in PBS. These brains were then postfixied with the same fixative containing 20% sucrose for cryoprotection.

**Magnetic Resonance Imaging**

The MRI experiments were performed on a 2.0-T GE CSI system (Bruker Instruments) equipped with shielded gradients capable of producing 20 G/cm. Rats were placed in the supine position with the heads inside a 5.5-cm-diameter birdcage radiofrequency coil. DWI and T2-weighted (T2W) scans were acquired at each imaging time point to monitor the infarct evolution. For DWI, an isotropic diffusion-weighted spin-echo sequence was used as described previously. Briefly, each set of diffusion-sensitizing gradients before and after the radiofrequency pulse had duration of 25 ms. B-values were 1300 s/mm² and 20 s/mm² for DWI and T2WI, respectively. From these images, pixel-by-pixel maps of the apparent diffusion coefficient (ADC) of water were calculated. Imaging parameters were as follows: 128×128 matrix; field of view, 50 mm; repetition time, 2.5 seconds; echo time, 80 ms; 1 average; 8 coronal slices; slice thickness, 1.5 mm; and interslice gap, 0.2 mm. Total imaging time

**Figure 1.** DWI and T2W MRI scans plus the corresponding ADC maps from 30 minutes and 5 hours after ischemia onset, showing complete reversal of the initial ischemic lesion (A). The extent of the DWI lesion is delineated by hand (red). At 5 hours, CV staining (B) and 5-hour DWI (A, lower left image) are normal, but the MAP2 stain shows loss of immunoreactivity (C, arrows).

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**Figure 2.** DWI and T2W MRI scans plus the corresponding ADC maps from 30 minutes and 5 hours after ischemia onset, showing partial reversal of the initial ischemic lesion (A). The extent of the DWI lesions is delineated by hand (red). At 5 hours, the CV (B) and MAP2 (C, arrows) stains show loss of reactivity. The extents of MAP2 and CV loss are similar and greater than the 5-hour DWI lesion.
Histopathology and Immunohistochemistry

Sections (25 μm thick) were cut on a cryostat at −20°C, then dried for 2 hours at room temperature and stored at −80°C. One set of brain sections for each animal was stained with cresyl violet (CV) to delineate regions of infarction, as evidenced by lack of staining. Immunohistochemical staining for a neuronal marker (murine monoclonal antibody against microtubule-associated protein 2 [MAP2]; No. HM-2, Sigma Chemical Co), an astrocyte marker (murine monoclonal antibody against glial fibrillary acidic protein [GFAP]; No. MAB360, Chemicon), and inducible HSP72 (StressGen murine monoclonal antibody; No. SPAR810) was performed on adjacent sections with the use of the avidin-biotin method according to standard protocols. Briefly, slices were treated with 0.5% Triton X-100, 0.03% hydrogen peroxide, and 0.1% bovine serum albumin in PBS for 20 minutes, then blocked for 1 hour with 5% milk at room temperature. Incubation with the primary antibody was performed overnight at 4°C or at room temperature for 1 hour. Dilutions of 1:500, 1:300, and 1:500 were used for the anti-MAP2, anti-GFAP, and anti-HSP72 antibodies, respectively. Slices were rinsed and treated for 1 hour with secondary biotinylated horse anti-mouse antibody (1:200, Vectastain ABC Elite kit, Vector Laboratories), preadsorbed to normal rat serum for 30 minutes. The sections were treated with Vectastain ABC reagent and DAB (Sigma), then counterstained with hematoxylin-eosin. Negative controls were run in parallel and were exposed to the same steps, except that the primary antibody was eliminated.

The paraformaldehyde-fixed tissue (7 days of survival after MCAO) was similarly prepared and stained with CV. Immunohistochemical staining was performed as described above, except that an antigen retrieval technique was used. Slides were placed in 0.1 mol/L citric acid into a microwave for 10 to 20 minutes, then allowed to cool to room temperature before staining.

Data Analysis

To determine the evolution of the ischemic injury, the MR images of each animal were matched to the histological sections. To consistently coregister the MR images with the histological image, we selected the 2 center slices from the imaging time points and matched them to the corresponding histological sections. The selected sections were at the level of the basal ganglia, including the anterior commissure, and 2 mm posterior to it, just anterior to the hippocampus. These 2 center sections also contained the bulk of the ischemic lesion, whereas the more anterior and posterior sections contained very little or no ischemic lesion. Lesions measured from the DWI scans were based on the knowledge that the ADC decreases as a function of time after ischemia onset. A prior study by Hoehn-Berlage et al11 showed that the ADC decreases progressively over the first hours after stroke onset (approximately 25%) with a corresponding cerebral blood flow decrease of approximately 18 mL/100 g per minute and ATP depletion. From the initial scans, regions where the ADC decreased by 25% corresponded to areas of increased signal on the DWI. Similar patterns were found for the 5-hour scans, except that the regions of high signal intensity were decreased by 30%. Therefore, hyperintense DWI lesions were identified by visual inspection and outlined manually. The lesion size was computed and expressed as the percentage of the ipsilateral hemisphere. Lesion areas were measured by an investigator blinded to the histology results.

From 2 corresponding histological sections, regions of infarction or lesion abnormality on the immunohistochemical stains were outlined by hand by an investigator blinded to the MRI results. Hemispheric lesion areas were measured with a computer-assisted image analysis system (MCID) and expressed as the percentage of the ipsilateral hemisphere.

Statistical analysis was performed with the use of SigmaStat software (SPSS). We used ANOVA/ANOVA for repeated measures followed by a multiple comparisons procedure to detect differences between groups. Significance was assessed at the P<0.05 level. Data are presented as mean±SEM.

Results

Five-Hour Group

After 30 minutes of MCAO, a distinct hyperintense ischemic lesion was detected on DWI, which regressed by 5 hours. Two of 5 animals showed complete reversal of the initial DWI lesion (Figure 1A), and the other 3 showed partial reversal (Figure 2A). Corresponding ADC maps and T2W within the territory of the reversal were also normal. The initial DWI hyperintensity was 46.5±0.1%; however, the lesion decreased to 4.7±1.6% by 5 hours after ischemia onset (Figure 3). CV-stained sections from the same brains (Figures 1B and 2B) showed loss of staining in a pattern similar to the 5-hour DWI scans. The CV lesion size was 2.4±1.1% and was not significantly different from the DWI lesion size from the same time point (Figure 3).

All animals, regardless of whether the 5-hour DWI was normal or not, showed loss of MAP2 staining in the core of the MCA territory at 5 hours (Figures 1C and 2C). The area of absent MAP2 staining was 14.9±3.1% and was significantly larger than the size of the DWI lesion at the same time point (5 hours after MCAO) (Figure 3). Regions of MAP2 loss were the same size as (Figure 2B and 2C) or larger than (Figures 1B, 1C, and 3) the lesions on the CV-stained sections. However, the CV and MAP2 lesion areas were still smaller than the initial DWI.

In areas where DWI was still positive at 5 hours, increased GFAP staining could be seen in a few reactive gemistocytic astrocytes (Figure 4A), although the majority of astrocytes was morphologically normal. However, in regions of DWI reversal, GFAP immunostaining within the striatum (Figure 4B) and cortex (Figure 4C) showed morphologically intact, nonreactive astrocytes. Astrocyte morphology in these re-

Figure 3. Comparison of DWI MRI scans with histochemistry. Lesion areas from the 5-hour scans are greatly reduced compared with the initial 0.5-hour DWI (DWI-0.5h; **P<0.01). The lesion size on the 5-hour DWI scans (DWI-5h) is different from the lesions on CV (CV-5h), but both are significantly smaller than the areas of MAP2 loss (MAP2-5h; *P<0.05). The extent of inducible HSP72 staining is similar to DWI-0.5h but is much greater than the lesions observed on DWI-5h, CV-5h, and MAP2-5h (**P<0.01).
regions was no different from the contralateral, nonischemic cortex (Figure 4D).

Sections immunostained for HSP72 predominantly stained neuronlike cells in striatal (Figure 4E) and cortical (Figure 4F) areas corresponding to increased signal in the initial DWI, which reversed by 5 hours. There was clear expression of HSP72 protein in brain regions outside of the lesion on the 5-hour DWI scan (Figure 4E and 4F) but not in the contralateral hemisphere (Figure 4G). Within areas of DWI nonreversal, some vessels were also immunopositive (Figure 4H). The area of HSP72 immunoreactivity was 45.1 ± 7.7% and was significantly different from the DWI lesion at 5 hours but was not significantly different from the initial DWI (Figure 3).

In cases in which reversal was incomplete, nonreversed tissue was confined to the striatum, where the severity of the ischemic insult may be greater, because of the presence of end vessels. Regardless, the histological findings of the reversed tissue at 5 hours, whether cortical or striatal, were similar.

Seven-Day Group
The initial DWI lesion after 30 minutes of ischemia was 44.1 ± 5% and was similar to the 5-hour survival group. By 3 hours after MCAO, the DWI lesion was markedly reduced to 5.8 ± 3.3% (Figure 5). In this group 2 animals showed complete DWI reversal, and the other animals showed a partial reversal of the initial DWI abnormalities. Nevertheless, in all cases the hyperintense region on DWI recurred 1 day after MCAO and measured 36.9 ± 3.7% (Figure 5). There was no DWI lesion at day 7, although infarcts were present on the CV sections (24.6 ± 3.4%). The 7-day T2W lesions corresponded to that delineated by CV and GFAP. GFAP staining was absent in the MCA territory, and the area of GFAP loss was no different from the area of infarction (Figure 6A and 6B). GFAP staining within the infarct border was increased in cells with thick processes (Figure 6C), whereas staining was decreased in the infarct center (Figure 6D). The lesions observed on CV and GFAP immunostains and T2W MRI at 7 days (Figure 5) were similar and were significantly smaller than the DWI lesions at 0.5 hour and 1 day after occlusion. However, they were larger than the DWI lesion at 3 hours. MAP2 immunohistochemistry at 7 days showed nonspecific staining within infarcted, necrotic cells and tissue (not shown).

Discussion
In this study we show that after a brief period of focal cerebral ischemia, there is histological evidence of ischemic injury
failure (reviewed by Siesjo et al.37). However, many of the alterations demonstrated by secondary energy loss due to mitochondrial dysfunction occurred in the setting of reperfusion, possibly mediated by free radical formation, apoptosis, or microvascular injury. This led some to believe that secondary ischemic insults.47 Therefore, much of the reversed tissue, especially the lesion observed by MAP2 immunostaining, we show that, despite a normal-appearing DWI, brain regions were already exhibiting histological and immunohistochemical evidence of irreversible damage.

We found that regions of immunoreactivity for HSP72 closely matched the initial DWI hyperintense region at 30 minutes. HSP72 is induced after a variety of stress stimuli, including cerebral ischemia,44,45 and is accepted as an indicator of cellular stress and injury.41,46 By 5 hours, neurons showed HSP72 immunostaining in the reversed area of the initially hyperintense DWI. Within regions of nonreversal, HSP72 was also observed in vessels, a finding that is consistent with prior observations that endothelial cells express HSP72 in brain regions exposed to the most severe insults.47 Therefore, much of the reversed tissue, especially neurons, still exhibited signs of metabolic stress. Our findings are in agreement with those of Mancuso and colleagues,24 who found that HSP72 mRNA was induced within regions of ADC decrease but persisted even after the ADC recovered.

Within regions of DWI reversal, GFAP immunostaining 5 hours after MCAO revealed morphologically normal, unreactive astrocytes throughout most of the ischemic and nonischemic hemispheres. Increased immunostaining in a few reactive astrocytes and retraction of astrocyte processes were observed only in regions where the 5-hour DWI remained positive. The appearance of morphologically normal astrocytes in regions of DWI reversal and the presence of some enlarged, reactive astrocytes in regions where DWI was positive suggest that astrocytes or other nonneuronal cell populations may contribute to the observed hyperintensities. However, others who examined a similar phenomenon in young animals found evidence for abnormal neuronal and astrocyte morphology during the ADC reversal. Miyasaka et al.48 found pronounced swelling of astrocytic perivascular endfeet and dendrites along with retracted neurons. They suggested that a balance between these shrunken neurons and swollen astrocytes may lead to an unchanged extracellular space with normal ADC values. Another study, using a similar ischemic hypoxia model,49 showed that 5 hours after insult, increased numbers of GFAP-immunoreactive astrocytes were present within the hippocampus during the period of ADC recovery along with rare terminal deoxynucleotidyl

Despite the reversal of the DWI, as previously reported by others,12,19–21 we found that signal intensity changes on DWI during ischemia reversed or were attenuated within hours of reperfusion. However, even with only 30 minutes of ischemia, DWI lesions recurred 12 to 24 hours later.22,25,26,28,30 DWI reversal was associated with recovery of the ADC and normal T2WI.28,31 These prior studies showed that this secondary ADC decrease was not associated with a failure of cerebral blood flow,31,32 although increased cerebral blood volumes have been described.26 Neurological recovery has been documented during the period of transient reversal, but worsening was not observed during the second ADC decrease.32 We show that during the period of transient DWI recovery there was widespread stress protein expression, indicating that the tissue was under metabolic stress. While stress protein expression does not necessarily imply irreversible damage, subregions of MAP2 loss were observed despite the normalization of the DWI scan. Because loss of MAP2 staining has previously been shown to indicate irreversible injury,33 the present findings suggest that DWI reversal in this instance did not reflect tissue recovery.

Recent studies have demonstrated delayed neuronal death34–36 or recurrent DWI lesions22,23,28,32 after mild cerebral ischemic insults. This led some to believe that secondary injury occurred in the setting of reperfusion, possibly mediated by secondary energy loss due to mitochondrial dysfunction, free radical formation, apoptosis, or microvascular failure (reviewed by Siesjo et al.37). However, many of the studies demonstrating delayed ischemic injury in different animal models concentrated on histological analysis between 12 hours and 3 days and not earlier. As we show here, the acute reversal of DWI lesions itself is not necessarily conclusive evidence of tissue recovery. In our study the DWI scans most closely paralleled the pattern of damage seen in the CV-stained histological sections. In 2 animals, ischemic regions on CV were visible as hyperintensities on the 5-hour scans. In contrast, we found a significantly larger area of lost immunoreactivity for MAP2 in comparison to the DWI and CV lesion among all animals regardless of the state of DWI reversal.

Loss of MAP2 immunostaining has received considerable attention as a marker for early ischemic changes in cerebral ischemia58,59 and is widely accepted as a sensitive marker of neurons reflecting irreversible structural damage.33,40,41 Dawson and Hallenbeck33 demonstrated close agreement of MAP2 loss and ischemic lesion size on hematoxylin-eosin after 1 hour, while others have suggested that MAP2 loss may be used as an indicator of viable neurons that have undergone irreversible damage.33,39,42 MAP2 loss also corresponded to ischemic hyperintensities observed on T2W 6 to 8 hours after occlusion,43 although we did not detect such T2W abnormalities here or in our previous report28; however, our ischemic insult was less severe in both studies. Although the intraischemic DWI at 30 minutes was also significantly larger than the lesion observed by MAP2 immunostaining, we show that, despite a normal-appearing DWI, brain regions were already exhibiting histological and immunohistochemical evidence of irreversible damage.
transferase–mediated dUTP biotin nick end-labeling (TUNEL)–positive neurons. Pronounced swelling and coexistence of lysed and apoptotic neurons were obvious at later time points when the DWI lesion returned and the ADC increased above normal. By 7 days we observed loss of GFAP staining, except within the infarct border, where staining was markedly increased. This is in agreement with other studies that examined GFAP immunoreactivity after cerebral ischemia. Astrocytes within the infarct core were few, and their processes appeared disrupted. The infarct determined from CV at 7 days corresponded to regions of absent GFAP immunoreactivity and indicated that both neuronal damage and glial damage were present at this late time point. Furthermore, lesion sizes from the T2W scans were similar to that of CV and loss of GFAP staining. Together, these studies suggest that the DWI signal likely reflects the state of tissue fluid balance but does not necessarily mean that the tissue itself is normal or recovered.

The results of this study are of potential clinical relevance, particularly because similar recovery of DWI in human stroke was recently described. Kidwell et al showed a reversal of diffusion abnormalities between 2.5 and 9 hours after thrombolysis. Interestingly, they also reported a secondary increase in diffusion lesions by day 7. Thus, the temporal course of ischemic changes in humans may resemble that in rats. Whether true neurological recovery can be documented with the use of DWI in humans is still unclear. Nevertheless, our data show that despite DWI recovery, neurons already show signs of irreversible damage. Thus, it will be important to identify other complementary measures of neurological recovery and not assume that all early DWI reversal necessarily reflects tissue salvage.

In conclusion, we demonstrate that after mild focal cerebral ischemia, DWI lesions reverse during early reperfusion, but the reversed tissue shows histological signs of cellular stress.

Figure 6. Histochemistry 7 days after ischemia onset. Loss of CV staining (A) and loss of GFAP (B) staining are similar. Higher-power views of the GFAP stains show increased staining in the infarct boundary (C), whereas within the infarct center (D), there is decreased immunoreactivity and staining only in disrupted cell processes. Bar=50 μm.
and irreversible damage. The precise source of the DWI signal has yet to be clarified, but our observations suggest that nonneuronal cells may play a more significant role than what was previously believed. Although DWI may not definitively reflect tissue salvage during early reperfusion, it is still a powerful method of detecting ischemic lesions at early time points. In the meantime, we believe that the results of our study warrant caution when relying solely on DWI as a surrogate to the neurological examination.

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