Identification of Variations in Blood-Brain Barrier Opening After Cerebral Ischemia by Dual Contrast-Enhanced Magnetic Resonance Imaging and T1sat Measurements

Tavarekere N. Nagaraja, PhD; Kishor Karki, MS; James R. Ewing, PhD; Richard L. Croxen, MS; Robert A. Knight, PhD

Background and Purpose—Variations in blood-brain barrier (BBB) opening after ischemia have been suggested by some tracer and magnetization transfer studies, although direct in vivo proof is still lacking. Contrast-enhanced magnetic resonance imaging (MRI) is also often used to visualize BBB damage in stroke. We hypothesized that MR contrast agents of different sizes enhance differently when BBB openings vary in size and that magnetization transfer alterations, measured by T1 sat, in these regions reflect such differences.

Methods—Male Wistar rats (300 g, n=7) were subjected to 3 hours of suture occlusion of the middle cerebral artery followed by reperfusion. Status of the BBB at 24 hours after the ictus was assessed first by Gd-DTPA (554 Da) MRI and then by Gd–bovine serum albumin linked to Evans blue (Gd-BSA-EB; 68 kDa) MRI for contrast enhancement; T1sat changes, cerebral blood flow, and blood-to-brain transfer constants (Ks) for the 2 contrast agents were measured. After MRI, rats were injected with fluorescent dextran and brains were studied by fluorescence microscopy.

Results—The Gd-BSA-EB–enhancing areas were always smaller (147±80 pixels) than those for Gd-DTPA (308±204 pixels) and were contained within the latter. The difference between the 2 areas was significant (P=0.024). Changes in T1sat were larger in Gd-BSA-EB–enhancing areas (ipsilateral to contralateral [I/C] 1.53±0.20) than in Gd-DTPA–enhancing areas (I/C 1.40±0.24, P=0.005). The differences in cerebral blood flow values between the 2 regions were not significant (P=0.62), but those for the Ks values of the 2 tracers were different (P=0.01 to 0.02). Excellent agreement between regions of Gd-BSA-EB enhancement and EB fluorescence was also observed.

Conclusions—These results substantiate earlier reports of regional differences in BBB opening after stroke and provide the first in vivo evidence for this phenomenon. They also support the possible use of T1 sat in quantifying stroke-induced graded BBB damage in the absence of contrast-enhanced MRI. (Stroke. 2008;39:427-432.)

Key Words: blood-brain barrier ■ brain drug delivery ■ brain imaging ■ middle cerebral artery ■ neurovascular unit

Reperfusion after focal cerebral ischemia has been shown to open BBB opening after stroke.1 Such opening is believed to lead to a different type of lesion than permanent arterial occlusion and to play a major role in reperfusion injury.2,3 Interest in acute BBB injury after stroke has recently been heightened since BBB opening was suggested as an avenue for therapeutic access to the brain.4,5 and the size of the BBB opening has been postulated to be predictive of impending hemorrhagic transformation.6 Therefore, maintaining barrier integrity, along with neuroprotective strategies, is suggested to be essential in attenuating ischemic brain injury.7 Evaluation of BBB damage and its response to therapy is also considered important in treating the complete neurovascular unit after cerebral ischemia.8 The opening of the BBB after ischemia is often looked at as an all-or-none phenomenon, but this may not be the case in at least some models of experimental stroke.9,10 Nonetheless, little is known about the physical nature of such openings, and no study so far has investigated them in vivo.

Magnetic resonance contrast agent (MRCA)–enhanced MR imaging (MRI) with Gd-DTPA is often used to evaluate BBB opening in stroke.11,12 However, with a molecular weight of ~550 Da and a Stokes-Einstein radius of ~7 Å, Gd-DTPA cannot differentiate between small and large BBB openings or, in other words, varied BBB damage. Owing probably to this limitation, the acute Gd-DTPA enhancement...
observed in experimental stroke models has tended to overestimate the area of later hemorrhagic transformation.\textsuperscript{11,13} Magnetization transfer (MT) is the exchange of spin magnetization between “free” (water) and “bound” (proton) pools, the latter associated with tissue macromolecules; changes in MT represent alterations in the amount of tissue water in contact with tissue macromolecules and other aspects of the interaction between the two. A few MRI studies that included noncontrast-based MT measurements have shown that $T_1$ under an off-resonance saturating radiofrequency field, or $T_{1\text{sat}}$, varies appreciably within the ischemic tissue and is well correlated in space and intensity with the degree of BBB leakage of the MRCA.\textsuperscript{14,15} MRCA-enhanced MRI permits the quantification of the blood-to-brain distribution of 2 different MRCA within a short duration of each other and thus, nearly concurrent estimates of BBB opening. On the basis of these observations, we hypothesized that (1) 2 MRCA of markedly different biophysical properties, Gd-DTPA and Gd-DTPA linked to bovine serum albumin (Gd-BSA), will distribute differently between blood and brain; and (2) that these differences will be correlated in degree and space with $T_{1\text{sat}}$. These hypotheses were tested in a rat model of transient middle cerebral artery (MCA) occlusion that has been shown to reliably result in BBB opening.\textsuperscript{16} After 3 hours of occlusion followed by reperfusion, the status of the BBB was sequentially examined at 24 hours, first with estimates of noncontrast-based $T_{1\text{sat}}$, then by Gd-DTPA, and finally by Gd-DTPA linked to BSA and Evans blue (Gd-BSA-EB; ~68 kDa; ~37 Å). Areas of enhancement for the 2 MRCA were measured and compared with the area of EB leakage visualized by fluorescence microscopy. Quantitative $T_{1\text{sat}}$ and blood-to-brain transfer constants ($K_i$) estimates and cerebral blood flow (CBF) values from the enhancing areas for the 2 MRCA were compared to each other.

**Materials and Methods**

**Preparation and Characterization of MRCA**

All chemicals used were purchased from Sigma-Aldrich-Fluka (Sigma Chemical Co., St. Louis, Mo) and used as received. The contrast agents Gd-BSA and Gd-DTPA were synthesized and characterized.\textsuperscript{17,18} The Gd-BSA preparation was then incubated with EB, an often-used, fluorescent, albumin-binding vital dye, and linked to it in a Gd-BSA/EB molar ratio of 1:7 to obtain Gd-BSA-EB. This additional step rendered the molecule with both paramagnetic and fluorescent properties and enabled direct comparison of MR images with those from fluorescence microscopy.

**Surgical Procedures**

All surgical procedures and experiments were performed under National Institutes of Health guidelines with the approval of the institutional animal care and use committee. Seven male Wistar rats (Charles River Laboratories, Wilmington, Mass) weighing ~300 g were used. Under halothane anesthesia, the right MCA was occluded with a 4.0 nylon filament as described in detail elsewhere.\textsuperscript{16,19} Three hours after MCA occlusion, the filament was withdrawn, initiating reperfusion for 21 hours.

**MRI and Fluorescence Microscopy**

At 24 hours after MCA occlusion, contrast-enhanced MRI was performed under halothane anesthesia according to published methods.\textsuperscript{15,20} In a 7 Tesla superconducting magnet (Magnex Scientific Inc, Abingdon, UK) interfaced to a Bruker Avance console (Bruker Biospin MRI, Billerica, Mass). One femoral artery and vein were cannulated with PE-50 catheters for blood pressure monitoring and MRCA administration during imaging, respectively. The rats were placed in a supine position in an acrylic holder with a nose cone for administering anesthetic gases and placed inside the magnet.

**Cerebral Blood Flow**

Estimates of CBF were obtained using an arterial spin-labeling technique. Labeling of inflowing arterial water protons was performed with an axial gradient of ±0.3 kHz/mm and a continuous-wave radiofrequency pulse at a power of 0.3 kHz and frequency offset of ±6 kHz, followed by a spin-echo (SE) sequence with a repetition time (TR)/echo time (TE) of 1050 ms/20 ms.\textsuperscript{21} Four averages of the image were acquired with the gradient polarities and the radiofrequency pulse frequency offsets reversed to remove any gradient asymmetries in the axial direction. The image was acquired over a 32-mm field of view and was reconstructed with a 64×64 image matrix.

$T_1$ and $T_{1\text{sat}}$ Measurements

The spin–lattice relaxation time, or $T_1$, data were acquired with an imaging variant of the T-one by multiple readout pulses sequence.\textsuperscript{22} Quantitative estimates of $T_{1\text{sat}}$ were also generated with this method.\textsuperscript{13} This was done by inserting 2 continuous-wave saturation radiofrequency pulses, with an 8-kHz frequency offset, into the Look-Locker (LL) sequence: the first (4.5 seconds) immediately before the inversion pulse and the second (40 ms) after signal acquisition. Initially, the longitudinal magnetization was inverted with an 8-ms nonselective, adiabatic, hyperbolic secant pulse. One phase-encode line of 32 small-tip-angle gradient-echo images (TR/TE=11 seconds/7.0 ms, 32-mm field of view, 128×64 matrix, 2-mm slice thickness) was acquired at 80-ms intervals after each inversion.

**BBB Permeability**

Estimates of $K_i$ were obtained with a $T_1$-weighted LL sequence (TR/TE=80 ms/4 ms, 128×64 matrix, 24 echoes, 5 slices each, 1.8 mm thick) to produce $T_1$ estimates.\textsuperscript{15,20} After baseline, precontrast SE $T_1$-weighted images (TR/TE=500 ms/7 ms, 32-mm field of view, 128×128 matrix, 13 slices each, 1.0 mm thick) and LL $T_1$ measurements were obtained, Gd-DTPA (80 μmol/kg IV) was injected, and a series of 10 LL $T_1$-weighted measurements were done at ~2.5-minute intervals for 25 minutes. A second set of SE, $T_1$-weighted images were acquired just after this series. The pre- and post-CA SE $T_1$-weighted images, which matched with the $T_{1\text{sat}}$ and CBF maps, were used to obtain a subtraction image that showed MRCA enhancement after leakage. The series of LL $T_1$ measurements were processed to obtain a permeability map for $K_i$ measurements. The same procedure was repeated for the Gd-BSA-EB (140 μmol/kg IV) injection after waiting for ~30 minutes for the Gd-DTPA signal to decay. Estimates of $K_i$ were produced with MRI Patlak plot methods.\textsuperscript{15,20}

Immediately afterward, the rats were removed from the magnet and injected intravenously with 1 mL fluorescein isothiocyanate–labeled dextran (2000 kDa; 50 mg/mL saline) that was allowed to circulate for 2 hours. They were then humanely killed by decapitation, and the brains were immersion-fixed in 10% buffered, neutral paraformaldehyde (VWR International, West Chester, Pa) for 48 hours. The fixed brains were cut into 60- to 70-μm-thick coronal sections in a Vibratome (Technical Products International Inc, St. Louis, Mo). The sections were individually mounted onto slides with Glycergel (Dako, Carpenteria, Calif) and coverslipped. The EB and fluorescein isothiocyanate–dextran fluorences were observed with a fluorescence microscope (Carl Zeiss, Thornwood, NY) equipped with appropriate filters.

**Image Analysis and Statistics**

The precontrast SE $T_1$-weighted image was subtracted from the postcontrast SE $T_1$-weighted images for both tracers to generate regions of interest with extravascular enhancement of Gd-DTPA and Gd-BSA-EB. The mean intensity plus 3 SDs taken from 3 random fields on the contralateral side was chosen to define the upper
boundary of normal gray-scale intensity to demarcate baseline pixels.\textsuperscript{23} The total area of BBB damage from each rat was calculated by counting the numbers of enhancing pixels above this baseline within the MR-defined ischemic lesion from each rat. The brain sections with EB leakage were digitized and reconstructed to show such regions. The total numbers of enhancing pixels from the Gd-DTPA and Gd-BSA-EB maps were compared with each other, and the Gd-DTPA and Gd-BSA-EB images were visually compared with EB fluorescence images. The CBF and T\textsubscript{1sat} values from the 2 enhancing areas from each rat were calculated\textsuperscript{15} and expressed as I/C. For each experiment, from the T\textsubscript{1}-weighted LL maps and Patlak plots, K\textsubscript{i} was also calculated for Gd-DTPA separately in Gd-DTPA– and Gd-BSA-EB–enhancing regions, and for Gd-BSA-EB in the Gd-BSA-EB–enhancing region. All values are reported as mean±SD. Two-tailed, paired Student’s t tests were used for statistical comparisons, and significance was inferred at P<0.05.

Results

The Gd-DTPA and Gd-BSA preparations were found to contain no unbound gadolinium or DTPA. At 7 T and 25°C, a 20% solution of Gd-BSA exhibited T\textsubscript{1} and T\textsubscript{2} times of 33.2 and 32.6 ms, respectively, whereas Gd-DTPA gave T\textsubscript{1} and T\textsubscript{2} times of 128.3 and 108.3 ms, respectively, at a comparable concentration.\textsuperscript{18}

All 7 rats lost some body weight during the 24-hour poststroke period. Average weight at surgery was 298±15 g and at 24 hours after the ictus, 258±11 g. The CBF I/C values in the stroke-affected brain regions were 0.64±0.15 in the Gd-DTPA– and 0.66±0.14 in the Gd-BSA-EB–enhancing areas (P=0.62).

Extravascular Gd-DTPA enhancement indicating BBB opening was observed in parts of the ischemic tissue in all rats. Subsequent enhancement of Gd-BSA-EB was also observed, although in every instance, this region was always smaller than that of Gd-DTPA (the Table) and contained within the latter (Figures 1A, 1B, 2A, and 2B). However, it should be noted that Gd-BSA-EB–enhancing pixels had also shown Gd-DTPA enhancement previously. However, for simplicity sake, they will be referred to as Gd-BSA-EB–enhancing areas. The number of pixels with Gd-DTPA enhancement was 308±204 and for Gd-BSA-EB, 147±80. The difference in the numbers of enhancing pixels for the 2 MRCAs was significant (P=0.02). For each animal, the changes in T\textsubscript{1sat} clustered in 2 ranges for these 2 areas (the Table). The normalized mean T\textsubscript{1sat} value from Gd-DTPA–enhancing areas was 1.40±0.24 and from those with Gd-BSA-EB (ie, both Gd-DTPA and Gd-BSA-EB) enhancement, 1.52±0.20 (P=0.005). Three different comparisons were made among the K\textsubscript{i} values (min\textsuperscript{-1}): (1) between Gd-DTPA K\textsubscript{i}s from the outer enhancing area (0.0016±0.001) and the smaller inner area, which showed Gd-BSA-EB enhancement extending outside the MRCAs was significant (P<0.005).

Table. MRI and Derived Values

<table>
<thead>
<tr>
<th>Rat No.</th>
<th>Pixels*</th>
<th>T\textsubscript{1sat} *</th>
<th>CBF*</th>
<th>Gd-DTPA K*</th>
<th>Gd-BSA-EB K†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>A</td>
<td>B</td>
<td>A</td>
</tr>
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<td>1.65</td>
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<tr>
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<td>219</td>
<td>102</td>
<td>1.34</td>
<td>1.46</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Total enhancing pixels, I/C ratios of T\textsubscript{1sat} values, CBF, and blood-to-brain K\textsubscript{i} estimates (min\textsuperscript{-1}) given individually for Gd-DTPA (A) and Gd-BSA-EB (B)–enhancing areas for the 7 rats studied.

*Two-tailed, paired t tests were used for statistical comparison of these data sets.
†Two-tailed, Student’s t tests were used for K\textsubscript{i} comparisons between Gd-DTPA and Gd-BSA-EB due to “noisy” Gd-BSA-EB K\textsubscript{i} data in experiment 2.
later on (0.0017±0.0010; P=0.59); (2) between Gd-DTPA $K_i$ from the outer Gd-DTPA area and Gd-BSA-EB $K_i$ (0.0004±0.0002; P=0.02); and (3) between Gd-DTPA $K_i$ from the inner area and Gd-BSA-EB $K_i$ (P=0.01).

Distributions of Gd-DTPA and Gd-BSA were reflected by quantitative T1sat maps. The regions with higher T1sat values were seen as brighter, hyperintense areas, and those with lower values, as darker gray (Figures 1C and 2C). Good visual agreement between the regions of Gd-BSA-EB enhancement and extravascular EB fluorescence was also observed in all cases (eg, Figures 1B and 1D, 2B and 2D). Cellular uptake of extravascular EB-labeled albumin in ipsilateral, BBB damaged regions was evident in the higher-magnification photomicrographs (data not shown).

Discussion

These data support the hypotheses and suggest regional differences in the degree of BBB opening within ischemia/reperfusion-damaged tissue and that T1sat may be sensitive to such differences, which may be the result of water shifts among blood and other tissue compartments and changes in associated tissue proteins.15,24 Supporting our first hypothesis, the smaller Gd-DTPA was observed to enhance across a much greater area than the larger Gd-BSA-EB. The alterations in T1sat were larger in the Gd-BSA-EB–enhancing area, supporting our second hypothesis. To the best of our knowledge, these are the first reported in vivo MRI observations of this phenomenon in stroke.

These results confirm the presence of diverse BBB openings in stroke, as suggested by earlier studies that used radiolabeled and fluorescent tracers of different sizes.9,10,25 One study reported that the transfer constants for sucrose (342 Da, 7 Å) were nearly 3 times higher than that of inulin (5000 Da, 13.5 Å) at 6 hours after a 10-minute period of global ischemia.25 Similar results were also found in a transient focal ischemia model at 24 hours after 2 hours of ischemia.10 Suggesting size-limited extravasation of plasma-borne substrates during acute reperfusion after 3 hours of MCA occlusion, EB was found to be leaking, whereas a large dextran (2000 kDa) was not.9 One study investigated the nature of BBB openings induced either osmotically or by hypertension26 and showed that tracers of different sizes such as sucrose, inulin, and fluorescent dextrans extravasated in a size-dependent fashion. The dissimilar MRCA leakage patterns observed in this study and the molecular size differences between Gd-DTPA and Gd-BSA-EB suggested variations in the size of stroke-induced BBB openings in this model.

Other contributing factors for the restricted distribution of Gd-BSA-EB may be the lower diffusivity of this larger CA in the relatively small and tortuous extracellular space of the brain27 and/or cellular uptake of extravascular albumin.28 The extracellular space in the ischemic brain can be further compressed by astrocytic swelling.29 However, as discussed next, concurrent changes in T1sat were larger in the Gd-BSA-EB–enhancing areas than in the Gd-DTPA-enhancing areas, suggesting that greater BBB damage was a major factor contributing to the observed differences in contrast enhancement. Therefore, we conclude that regions with Gd-BSA-EB enhancement may have larger openings and hence, greater BBB damage.

One possible shortcoming of this study was that changing the order of Gd-DTPA and Gd-BSA-EB administration was not attempted. This experimental design was not possible because unlike Gd-DTPA (plasma half-life of ≈18 minutes), albumin and albumin-bound substrates have a plasma half-life of ≈3 hours and even with a bolus injection, are known to maintain relatively constant plasma levels for several hours, by which time BBB pathology might change. These factors would not have allowed Gd-DTPA imaging in rapid succession with Gd-BSA-EB.

These differences in relaxivity, plasma half-life, and distribution patterns of the 2 CAs are also suspected to have resulted in the different enhancement patterns. However, use of the Patlak plot takes into account the blood levels of the tracer in question in estimates of $K_i$, thus facilitating direct quantitative comparisons irrespective of tracer properties.

Opening of the BBB has been noted in several disorders. In a growing tumor, it is believed to indicate ongoing angiogenesis, and its absence is considered an indicator of treatment efficacy. On the contrary, in acute stroke, transient BBB opening has also been reported.4,30 Such openings have been suggested as predictors of looming pathology6 and also as possible conduits to deliver therapeutics to the injured brain, which is otherwise not very accessible.3,5,31 However, the putative drugs may be limited by their own effective diameters (eg, presence of hydration rings that increase the native molecular size) and/or the size of available openings. Therefore, quantitative, noninvasive analysis of the size of BBB openings may help determine an optimal drug delivery vehicle to the injured brain via transient BBB openings. Characterizing the severity and nature of neuronal injury...
associated with such varied BBB openings may help determine their role in predicting impending tissue damage, as suggested by some investigators.6

Contrast-enhanced detection of BBB damage is dependent on the delivery of the MRCA to the affected region(s). In stroke, CBF is the first parameter to be affected, and in areas of very low CBF, BBB damage may go undetected due to inefficient MRCA delivery. In fact, it has been shown that owing to the steep drop in CBF, the severity of ischemic damage may sometimes be inversely related to the degree of MR contrast enhancement.32 Additionally, as noted before, Gd-DTPA enhancement alone may not be a spatially accurate predictor of ensuing hemorrhagic transformation after stroke.11,13 Therefore, noncontrast-based MRI techniques as surrogate markers to detect BBB injury after stroke are also being sought. Some MT parameters have shown promise in this regard. In particular, T\textsubscript{1sat} was shown to be sensitive to changes in free and protein-bound water pools as a result of BBB damage and consequent edema.14,24,33 Significant BBB damage after stroke, often predictive of impending hemorrhagic transformation, was reported to be indicated by higher I/C ratios of T\textsubscript{1sat}.24 In the present study, I/C ratios of T\textsubscript{1sat} were incrementally elevated in Gd-BSA-EB–enhancing regions, apparently with more BBB damage, when compared with Gd-DTPA–enhancing regions or regions with less BBB damage. These observations provide further evidence for the predictive ability of T\textsubscript{1sat} to track variations in BBB damage and suggest its possible clinical use to quantify graded BBB damage in the absence of contrast-enhanced MRI.

Summary
Opening of the BBB after stroke and reperfusion is a well-known phenomenon. Such opening is varied and may even be biphasic. Neuroprotection offered by some therapies is suggested to be partially due to vasoprotective actions.7 Moreover, vasoprotective drugs are being sought for combination therapy with tissue plasminogen activator to make the latter safer.34 Yet quantitative evaluation of BBB opening after stroke and its response to putative therapies is not being done regularly. It seems crucial to perform such studies to understand the effects of temporal and spatial changes in BBB permeability on the progression of stroke injury, in mediating the therapeutic efficacy of putative neuroprotective agents, and to evaluate vasoprotective drugs for potential combination therapy with tissue plasminogen activator. Noninvasive, repeatable measures such as T\textsubscript{1sat} may be useful for such purposes.

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Disclosures
None.

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