Diffusion-Weighted Magnetic Resonance Imaging Confirms Marked Neuroprotective Efficacy of Albumin Therapy in Focal Cerebral Ischemia

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Background and Purpose—We have recently shown high-dose human serum albumin therapy to confer marked histological protection in experimental middle cerebral artery occlusion (MCAo). We have now used diffusion-weighted magnetic resonance imaging (DWI) in conjunction with morphological methods to expand our understanding of this therapeutic approach.

Methods—Physiologically controlled Sprague-Dawley rats received 2-hour MCAo by the modified intraluminal suture method. Treated rats received 25% human serum albumin solution (1% by body weight) immediately after the MCA was reopened. Vehicle-treated rats received saline. Computer-based image averaging was used to analyze DWI data obtained 24 hours after MCAo and light-microscopic histopathology obtained at 3 days. In a matched series, plasma osmolality and colloid oncotic pressure, as well as brain water content, were determined.

Results—Albumin therapy, which lowered the hematocrit on average by 37% and raised plasma colloid oncotic pressure by 56%, improved the neurological score throughout the 3-day survival period. Within the ischemic focus, the apparent diffusion coefficient (ADC) computed from DWI data declined by 40% in vehicle-treated rats but was preserved at near-normal levels (8% decline) in albumin-treated rats (\( P < 0.001 \)). Albumin also led to higher ADC values within unlesioned brain regions. Histology revealed large consistent cortical and subcortical infarcts in vehicle-treated rats, while albumin therapy reduced infarct volume at these sites, on average, by 84% and 33%, respectively. Total infarct volume was reduced by 66% and brain swelling was virtually eliminated by albumin treatment. Microscopically, while infarcted regions of vehicle-treated rats had the typical changes of pannecrosis, infarcted zones of albumin-treated brains showed persistence of vascular endothelium and prominent microglial activation, suggesting that albumin therapy may help to preserve the neuropil within zones of residual infarction.

Conclusions—These findings confirm the striking neuroprotective efficacy of albumin therapy in focal cerebral ischemia and reveal that this effect is associated with DWI normalization and a mitigation of pannecrotic changes within zones of residual injury. (Stroke. 1998;29:2587-2599.)

Key Words: colloid oncotic pressure ■ diffusion ■ image processing, computer-assisted ■ microglia ■ middle cerebral artery occlusion ■ rats

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eomodulating agents—chiefly, the dextrans—have been investigated for many decades as a potential therapy for ischemic stroke. The prime rationale for this approach is that cerebral blood flow (CBF) varies inversely with hematocrit and whole-blood viscosity,\(^1\) and hemodilution has been shown to increase CBF of both the normal and ischemic brain, either by decreasing blood viscosity or by vasodilation in response to diminished oxygen delivery.\(^2\) Despite certain encouraging experimental results, several clinical trials of hemodilution in ischemic stroke have nonetheless proven negative or inconclusive.\(^3-5\) Each of these studies has been criticized because of the relatively late patient entry time and/or the modest degree of hemodilution achieved. By contrast, previous studies have directed only scant attention to the importance of the specific hemodiluting agent itself.

Albumin, an endogenous plasma protein with important physiochemical properties,\(^6\) has commonly been regarded as an alternative hemodiluting agent to dextran\(^7,8\) but until recently has not been rigorously evaluated for its anti-ischemic neuroprotective efficacy. Cole et al\(^9\) reported a positive effect of 5% albumin in reducing ischemic brain injury, an action that was augmented by pharmacological hypertension.

Received April 3, 1998; final revision received August 18, 1998; accepted September 2, 1998.

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Matsui et al.\textsuperscript{10} noted diminished brain edema and infarct volume in rats with middle cerebral artery occlusion (MCAo) treated with concentrated (25\%) albumin begun after 30 minutes of ischemia.

In a recent study we administered 20\% human serum albumin to rats at the onset of recirculation after a 2-hour period of MCAo and documented a substantial diminution of infarct volume together with a marked reduction of brain edema.\textsuperscript{11} The latter effect suggested that albumin therapy might strikingly modify the water homeostasis of the ischemic brain. Thus, we designed the present study, using a highly reproducible model of focal cerebral ischemia,\textsuperscript{12} to explore this mechanism by means of diffusion-weighted magnetic resonance imaging (DWI), a method very sensitive to parenchymal water alterations.\textsuperscript{13} No prior studies have used MRI to evaluate the effects of albumin treatment on brain ischemia. In addition, we confirmed the therapeutic effect of albumin by histopathological quantitation of infarct size, immunochemical evaluation of activated microglia, and neurobehavioral assessment. In a matched series, we also assessed plasma osmolality and colloid osmotic pressure, as well as brain water content.

Materials and Methods

Surgical Preparation

Twenty-two adult male Sprague-Dawley rats (weight, 330 to 400 g; Crl:CD (SD)BR strain, Charles River Laboratories, Wilmington, Mass) were used in these studies. They were fasted overnight but allowed free access to water. Study protocols were approved by the Animal Care and Use Committee of the University of Miami. After administration of atropine sulfate (0.5 mg/kg IP), anesthesia was induced with 3.5\% halothane in a mixture of 70\% nitrous oxide and oxygen. Rats were orally intubated, immobilized with pancuronium bromide (0.6 mg/kg IV), and mechanically ventilated. Temperature probes were inserted into the rectum and the left temporalis muscle, and separate heating lamps were used to maintain rectal and cranial temperatures at 37.0 to 37.5°C (Mon-a-therm 7000; Mallinckrodt, Inc). The right femoral artery and vein were catheterized for continuous blood pressure monitoring and periodic blood sampling for arterial gases, pH, hematocrit, and plasma glucose (15 minutes before MCAo; at 15, 90, and 110 minutes after MCAo; and 15 minutes after MCA suture removal). Mean arterial pressure was measured with the use of an indwelling femoral arterial catheter connected to a precalibrated Statham pressure transducer (model P23XL; Viggo-Spectramed Inc) and was recorded continuously (model RS3400 polygraph; Gould, Inc). Serial measurements were made of arterial blood gases and pH (model ABL 330; Radiometer America, Inc) and plasma glucose (model 2300 Stat; Yellow Springs Instrument Co, Inc). During the 3-day survival period, rectal temperature, body weight, mean arterial pressure, and hematocrit were monitored periodically.

Middle Cerebral Artery Occlusion

The right MCA was occluded for 2 hours by our modification\textsuperscript{12} of the intraluminal suture method of Zea Longa et al.\textsuperscript{14} In brief, the right common carotid artery was exposed through a midline neck incision and dissected free of surrounding nerves, the occipital branches of the external carotid artery were coagulated, and the pterygopalatine artery was ligated. A 4-cm length of 3-0 monoflament nylon suture was then inserted through the proximal external carotid artery into the internal carotid artery and MCA, a distance of 19 to 20 mm from the common carotid artery bifurcation according to the animal’s weight, thereby occluding the MCA. Before use, the tip of the suture was heat-blunted, and a 20-mm distal segment of the suture was coated with poly-L-lysine solution (0.1\% wt/vol) and dried at 60°C for 1 hour; this coating procedure enhances the reproducibility of the resulting infarct.\textsuperscript{12} After suture placement, the neck incision was closed, and animals were allowed to awaken from anesthesia. At 60 minutes after MCAo, they were tested on a standardized neurobehavioral battery to confirm the presence of a neurological deficit.\textsuperscript{12} Animals that did not demonstrate a right upper extremity paresis were excluded from further study. After 2 hours of MCAo, rats were reanesthetized, temperature probes were reinserted, and the intraluminal suture was carefully removed. Sham-operated animals underwent all procedures except for MCAo.

Neurological Evaluation

Behavioral tests were performed in all rats before MCAo, during occlusion (at 60 minutes), and daily for 3 days after MCAo. The battery consisted of the postural reflex test to examine upper body posture while the animal is suspended by the tail\textsuperscript{15} and the forelimb placing test to examine sensorimotor integration in forelimb placing responses to visual, tactile, and proprioceptive stimuli.\textsuperscript{16} Neurological function was graded on a scale of 0 to 12 (normal score=0, maximal score=12), as previously described.\textsuperscript{12}

Treatment Groups

In each series described below, albumin-treated rats with MCAo or sham MCAo received human serum albumin (Alpha Therapeutic Corp; 25\% solution), which was administered intravenously (1\% of body weight) at a constant rate over 3 minutes immediately after suture removal in MCAo rats or at the corresponding time point in sham MCAo animals. Vehicle-treated rats received an intravenous infusion of a comparable volume of 0.9\% sodium chloride.

Study Protocols

Two protocols were used. In series 1, rats were studied by MRI 24 hours after MCAo or sham MCAo (MCAo group: albumin treated, n=3; saline treated, n=3; sham MCAo group: albumin treated, n=3; saline treated, n=3). These rats were then killed at 3 days for histopathology. In series 2, only histopathology (3-day survival) was assessed (MCAo group: albumin treated, n=6; saline treated, n=4).

Magnetic Resonance Imaging

Rats of series 1 were imaged 24 hours after MCAo or sham MCAo on a 1.5-T whole-body MRI system (EDGE, Picker International Inc). This unit has self-shielded gradient coils with 16 mT/m peak gradient strength and a 20 mT/m per second slew rate. A quadrature body coil was used as a transmitter, and a specially designed 4-cm-diameter single-loop coil was used as a receiver to provide high-resolution cranial images. In preparation for MRI, rats were anesthetized with chloral hydrate (300 mg/kg) and were placed in a home-built acrylic plastic stereotoxic holder. The head was positioned within the radio-frequency coil, and the coil was then centered in the magnet. The body temperature was monitored and maintained at 36.5°C to 37.5°C during the MRI study with a gel-filled heating pad (Rubbermaid Specialty Products Inc). The MRI procedure lasted ~50 minutes.

Axial spin-echo localized images were acquired for accurate positioning of subsequent slices. The field of view was 40 mm, and slice thickness was 2 mm. Diffusion imaging was performed with a spin-echo technique, with diffusion-encoding gradients applied on either side of the 180-degree radio-frequency pulse.\textsuperscript{17,18} Coronal diffusion-weighted images were obtained with diffusion encoding applied along the slice select axis. One image was acquired without the diffusion-encoding gradient (“reference image”), and 4 diffusion-weighted images with different b values (205, 410, 615, and 825 s/mm\textsuperscript{2}) were used to obtain calculated apparent diffusion coefficient (ADC) images (image parameters: repetition time, 1000 ms; echo time, 130 ms; excitations, 4; 128×128 image matrix). ADC values were computed on a pixel-by-pixel basis by using a linear regression algorithm to fit a straight line to the logarithm of signal intensity on the reference image and the 4 diffusion-weighted scans with different b values.
TABLE 1. Physiological Variables

<table>
<thead>
<tr>
<th></th>
<th>Series 1</th>
<th></th>
<th>Series 2</th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>MCAo-Saline (n=3)</td>
<td>MCAo-Albumin (n=3)</td>
<td>Sham-Saline (n=3)</td>
<td>Sham-Albumin (n=3)</td>
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<td>15 min before MCAo</td>
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<td>Cranial temperature, °C</td>
<td>37.2±0.03</td>
<td>37.3±0.03</td>
<td>37.3±0.03</td>
<td>37.0±0.22</td>
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<td>Rectal temperature, °C</td>
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<td>37.5±0.03</td>
<td>37.6±0.10</td>
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<td>7.42±0.01</td>
<td>7.45±0.01</td>
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<td>PaO2, mm Hg</td>
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<td>89.7±5.7</td>
<td>117.1±17.7</td>
<td>104.9±8.1</td>
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<td>PaCO2, mm Hg</td>
<td>39.1±1.6</td>
<td>40.8±1.5</td>
<td>38.3±1.1</td>
<td>38.4±2.6</td>
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<td>MABP, mm Hg</td>
<td>100±6</td>
<td>90±6</td>
<td>105±4</td>
<td>120±0</td>
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<tr>
<td>Plasma glucose, mg/dL</td>
<td>120±18</td>
<td>125±8</td>
<td>116±16</td>
<td>138±8</td>
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<td>Hematocrit, %</td>
<td>40.0±1.2</td>
<td>38.3±0.9</td>
<td>43.3±0.8</td>
<td>41.7±0.9</td>
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<tr>
<td>15 min after MCAo</td>
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<td></td>
<td></td>
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<tr>
<td>Cranial temperature, °C</td>
<td>37.1±0.03</td>
<td>37.1±0.06</td>
<td>37.6±0.32</td>
<td>37.0±0.17</td>
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<td>Rectal temperature, °C</td>
<td>37.3±0.01</td>
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<td>37.7±0.37</td>
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<td>pH</td>
<td>7.38±0.01</td>
<td>7.43±0.02</td>
<td>7.44±0.02</td>
<td>7.45±0.01</td>
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<td>PaO2, mm Hg</td>
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<td>107.9±2.1</td>
<td>131.0±19.2</td>
<td>104.7±6.8</td>
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<td>PaCO2, mm Hg</td>
<td>39.1±0.3</td>
<td>37.7±0.8</td>
<td>39.0±1.5</td>
<td>38.1±1.3</td>
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<tr>
<td>MABP, mm Hg</td>
<td>108±2</td>
<td>120±8</td>
<td>105±4</td>
<td>123±2</td>
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<tr>
<td>Plasma glucose, mg/dL</td>
<td>116±11</td>
<td>115±6</td>
<td>109±19</td>
<td>127±5</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>39.7±2.9</td>
<td>41.0±2.0</td>
<td>43.7±0.7</td>
<td>37.0±3.2</td>
</tr>
<tr>
<td>15 min after recirculation</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cranial temperature, °C</td>
<td>37.3±0.07</td>
<td>37.3±0.03</td>
<td>37.5±0.03</td>
<td>37.3±0.07</td>
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<td>Rectal temperature, °C</td>
<td>37.2±0.04</td>
<td>37.3±0.03</td>
<td>37.5±0.03</td>
<td>37.3±0.09</td>
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<td>MABP, mm Hg</td>
<td>87±3</td>
<td>83±2</td>
<td>85±2</td>
<td>82±4</td>
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<tr>
<td>Hematocrit, %</td>
<td>41.0±3.6</td>
<td>28.3±1.2*</td>
<td>42.3±0.7</td>
<td>26.3±2.6*</td>
</tr>
<tr>
<td>1 d after recirculation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rectal temperature, °C</td>
<td>37.4±0.04</td>
<td>37.5±0.06</td>
<td>37.5±0.04</td>
<td>37.4±0.08</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>38.3±3.7</td>
<td>37.3±2.9</td>
<td>42.0±0.6</td>
<td>42.0±0.6</td>
</tr>
</tbody>
</table>

Data are mean±SEM. MABP indicates mean arterial blood pressure; series 1, MRI+histology; series 2, histology only. *Different from MCAo-Saline and Sham-Saline groups (P<0.05, Student’s t test).

Five contiguous slices, each 2 mm thick, were obtained with a 50-mm field of view. These corresponded to bregma levels +2.2, +0.2, −1.8, −3.8, and −5.8 mm. For each of these slices, the reference image was used to assess the topography of infarction. This was chosen in lieu of classic T2-weighted imaging to minimize the total study duration.

TABLE 2. Osmolality and Colloid Oncotic Pressure

<table>
<thead>
<tr>
<th></th>
<th>MCAo-Saline (n=4)</th>
<th>MCAo-Albumin (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 min before MCAo</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma osmolality, mOsm/kg</td>
<td>278.2±2.7</td>
<td>281.6±1.0</td>
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<tr>
<td>Plasma colloid oncotic pressure, mm Hg</td>
<td>17.8±0.2</td>
<td>18.4±1.5</td>
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<tr>
<td>15 min after treatment</td>
<td></td>
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<tr>
<td>Plasma osmolality, mOsm/kg</td>
<td>276.7±0.6</td>
<td>277.8±5.3</td>
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<tr>
<td>Plasma colloid oncotic pressure, mm Hg</td>
<td>16.1±1.1</td>
<td>25.1±1.0*</td>
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<tr>
<td>1 d after recirculation</td>
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<td></td>
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<tr>
<td>Plasma osmolality, mOsm/kg</td>
<td>283.2±2.3</td>
<td>280.9±3.2</td>
</tr>
<tr>
<td>Plasma colloid oncotic pressure, mm Hg</td>
<td>19.7±2.2</td>
<td>24.7±2.1</td>
</tr>
</tbody>
</table>

Data are mean±SEM. *Different from MCAo-Saline group (P<0.05, Student’s t test).

Histological Assessment of Infarction and Edema Volume

Animals were allowed to survive for 3 days after MCAo or sham MCAo. Brains were then perfusion-fixed as previously described with a mixture of 40% formaldehyde, glacial acetic acid, and methanol (1:1:8 by volume), and brain blocks were embedded in paraffin. Ten-μm-thick sections were cut in the coronal plane and stained with hematoxylin and eosin. To quantify infarct volume, histological sections were digitized at 9 standardized coronal levels by means of a charge-coupled device–based camera (Xillix Technologies Corp) interfaced to an MCID image analysis system (Imaging Research), from which data were exported to a DEC-Alpha workstation (Digital Equipment Corp) for processing. An investigator blinded to the experimental groups then outlined the zones of infarction (which were clearly demarcated) as well as the outlines of the left and right hemispheres on each section. Infarct volume was calculated as the integrated product of cross-sectional area and intersection distance. The infarct volume of each rat was corrected for swelling of the ischemic hemisphere by applying the following formula: Corrected Infarct Volume = Left Hemisphere Volume − (Right Hemisphere Volume − Measured Infarct Volume). Brain swelling was determined as the percent difference in brain volume between the 2 hemispheres.
Image Processing

DWI and reference MR images in individual rats were exported to a DEC-Alpha workstation for further processing. Reference MR images at each slice level were converted to a binary format by using region-of-interest routines to measure the average intensity of the left hemisphere of the slice (omitting high-signal cerebrospinal fluid–containing regions) and applying a mean±2 SD threshold criterion. At each level, DWI data from individual rats of each subgroup were mapped into a standardized coronal contour based on the atlas of Zilles and were averaged by the method of disparity analysis developed by m22 to yield a quantitative image of mean ADC value at each level.

The digitized binary images of histological infarction in individual rats were also mapped by disparity analysis into a common atlas template at each coronal level studied. Pixel-by-pixel summation of these data yielded maps depicting, for each subgroup, the relative frequency of infarction.12,23

Immunohistochemistry

Selected deparaffinized brain sections were reacted for the histochemical visualization of activated microglia with peroxidase-labeled isocistin-B4 from Bandeiraea simplicifolia (GSA I-B4).24 These sections were incubated with 1.5% hydrogen peroxide diluted with methanol for 20 minutes, followed by a 10-minute washing in PBS. Slides were incubated with 0.1% Triton X-100 in PBS for 15 minutes, then with isocistin B4 (Sigma Chemical Co) for 2 hours. Slides were washed with PBS and stained with 3,3’-diaminobenzidine tetrachloride and hydrogen peroxide for 2 to 3 minutes.

Measurement of Plasma Osmolality, Plasma Colloid Oncotic Pressure, and Regional Brain Water Content

In 2 separate groups of rats (saline treated, n=4; albumin treated, n=4), plasma osmolality was measured by an osmometer (model 4400, Wescor, Inc) at 15 minutes before MCAo and at 15 minutes and 24 hours after treatment.

In these same rats, regional brain water content was also determined at 24 hours after MCAo by the wet weight/dry weight method, which we have previously described in detail.25 Samples of brain tissue weighing ~20 mg were taken from the lateral frontoparietal neocortex and striatum of both hemispheres. Percent water content was calculated by the following equation: % Water Content=[(Wet Weight−Dry Weight)/Wet Weight]×100.

Statistical Analysis

Physiological variables, infarct volumes, and percentage of brain swelling were compared in saline- and albumin-treated rats by Student’s t tests. Infarct areas, brain swelling at various coronal levels, and neurological scores were analyzed by repeated-measures ANOVA with post hoc Bonferroni tests. Pixel-based average ADC data in saline- versus albumin-treated subgroups were compared by Kolmogorov-Smirnov 2-sample tests.26 Infarct frequency maps in saline- and albumin-treated rats were compared on a pixel-by-pixel basis by the Fisher exact test.26

Results

Physiological Variables

Rectal and cranial (temporalis muscle) temperatures, arterial blood pressure, plasma glucose, and blood gases showed no significant differences among groups (Table 1). The hematocrit in the saline-treated groups averaged 39.9±0.7% (baseline) and 40.7±1.4% (15 minutes after saline infusion). Hematocrit in the albumin-treated groups was 38.8±0.7% at baseline and was reduced to 24.4±1.4% by albumin treatment (P<0.05; Table 1). By 24 hours, the hematocrit had returned to normal.

Plasma osmolality and colloid oncotic pressure are presented in Table 2. Plasma osmolality was not affected by albumin treatment. By contrast, the plasma colloid oncotic pressure was significantly higher at 15 minutes in animals treated with albumin than in the saline-treated group. Plasma colloid oncotic pressure also tended to be higher at 24 hours in albumin-treated rats compared with the saline-treated group, but this difference did not reach statistical significance (Student’s t test).

Treatment with albumin did not change the water content of the right (ischemic) cortex compared with the saline group (mean±SEM, 82.5±2.6% versus 82.2±1.2%, respectively). However, water content of the left (nonischemic) cortex was reduced by prior albumin therapy (77.3±0.4% versus 79.4±0.3%, respectively; P=0.01). The water content of the right (ischemic) striatal area was 2.5% less in the albumin-treated group than in the control group (81.9±3.8% versus 84.2±1.1%, respectively), but this difference did not reach statistical significance (Student’s t test). Left striatal water content was the same in albumin- and saline-treated rats (76.7±0.9% versus 76.3±1.1%, respectively).

Neurobehavioral Deficits

Contralateral forelimb placing deficits were clearly present at 60 minutes after MCAo in all rats (Figure 1). Albumin significantly improved the neurological score compared with saline at 24, 48, and 72 hours after MCAo (Figure 1).

Reference MR Images

In both saline- and albumin-treated rats with sham MCAo, reference MR images appeared entirely normal, without evidence of focal lesions. In saline-treated rats with MCAo, extensive confluent hyperintense lesions involved the dorso-lateral and lateral regions of frontoparietal neocortex of the right hemisphere, as well as the subjacent caudoputamen (Figure 2A). By contrast, albumin-treated rats showed considerably smaller hyperintense zones affecting the caudoputamen but largely sparing the overlying cortex (Figure 2A).

Apparent Diffusion Coefficient

ADC images in sham-operated rats appeared homogeneous, but ADC values were noticeably higher in albumin-treated
than in saline-treated rats (Figure 2B). In saline-treated rats with MCAo, conspicuous zones of reduced ADC values were present in the neocortex and caudoputamen of the ipsilateral hemisphere; these regions corresponded to the hyperintense-lesioned zones of the reference MR images (Figure 2C). By comparison, in albumin-treated rats with MCAo, both the magnitude and the topographic extent of this ADC decline were considerably less than in their saline-treated counterparts (Figure 2C).

ADC was further analyzed by a pixel-based approach that separately considered ADC changes in “positive” (ie, signal intensity $>$2 SD of mean left hemisphere value) versus “negative” pixels (ie, signal intensity $\leq$2 SD of left hemisphere value) of the corresponding reference MR image. Table 3 and Figures 3 and 4 summarize this analysis. In rats with MCAo, prior albumin therapy led to a remarkable preservation of nearly normal ADC values even within lesioned (ie, reference-MR–positive) brain areas. Thus, in saline-treated rats, prior MCAo produced a 40% decrease in mean ADC value within reference-MR–positive pixels; in marked contrast, ADC values in albumin-treated rats with MCAo fell, on average, by only 8% below control (Table 3). The difference in the distribution of ADC values in lesioned

### Table 3. Apparent Diffusion Coefficient

<table>
<thead>
<tr>
<th></th>
<th>Saline Treated (n=3)</th>
<th>Albumin Treated (n=3)</th>
<th>$P^*$</th>
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<tbody>
<tr>
<td>R hemisphere, ref+</td>
<td>0.608±0.071</td>
<td>1.132±0.043</td>
<td>$&lt;0.001$</td>
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<tr>
<td>R hemisphere, ref−</td>
<td>0.742±0.086</td>
<td>0.996±0.115</td>
<td>$&lt;0.05$</td>
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<tr>
<td>L hemisphere, ref−</td>
<td>0.826±0.084</td>
<td>1.049±0.023</td>
<td>$&lt;0.05$</td>
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<tr>
<td>Sham rats</td>
<td>0.609±0.064</td>
<td>1.233±0.124</td>
<td>$&lt;0.05$</td>
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</tbody>
</table>

Values are mean±SD, expressed in $10^{-3}$ mm$^2$/s. Ref+ indicates pixels whose intensity on reference MR image $>$2 SD of mean left hemisphere value; ref−, pixels whose intensity on reference MR image $\leq$2 SD of mean left hemisphere value.

*Kolmogorov-Smirnov 2-sample test.*
(ie, reference-MR–positive) pixels of MCAo rats treated with saline versus albumin was highly significant ($P < 0.001$; Figure 3).

In comparison to sham brains, MCAo also led to moderately reduced ADC values in the unlesioned (ie, reference-MR–negative) zones of the right hemisphere as well as in left hemisphere pixels, but these decreased ADC values tended to be more prominent (26% and 18%, respectively) in saline-treated rats than in the albumin-treated group (19% and 15%, respectively) (Table 3). In each of these unlesioned regions of MCAo brains, and as well in sham MCAo brains, prior albumin treatment led to a significant rightward shift of the ADC distribution curves compared with the data from saline-treated rats (Figure 4). Albumin administration in sham MCAo rats was associated with a 22% increase in mean ADC value compared with animals receiving saline ($P < 0.05$; Table 3).

**Histopathology**

All animals survived uneventfully. Histological examination of the brains of saline-treated rats with MCAo followed by 72-hour survival showed large consistent zones of infarction involving the frontoparietal neocortex and underlying caudoputamen. These infarcted regions showed pancellular necrosis as well as dense areas of eosinophilic, shrunken neurons along the infarct margins. By contrast, albumin-treated rats showed markedly smaller cortical infarcts and somewhat reduced zones of basal-ganglionic infarct as well. Table 4 presents infarct volumes and percent brain swelling separately for series 1 and 2, and Figure 5 depicts the rostrocaudal distribution of cortical (Figure 5A) and subcortical (Figure 5B) infarct areas in saline- and albumin-treated rats with MCAo or sham occlusion. (Reference-MR–negative refers to those pixels of the reference-MR image whose values did not exceed 2 SD of mean left hemisphere values; $n=3$ rats per group.) The ADC distribution of albumin-treated rats is significantly rightward-shifted compared with saline-treated rats both in the reference-MR–negative pixels of the right (ipsilateral) hemisphere of rats with MCAo, as well as in the left hemisphere of both MCAo rats and sham-occluded animals ($P < 0.05$, Kolmogorov-Smirnov 2-sample test).

Figure 3. Distribution of ADC values in reference-MR–positive pixels of the right hemisphere of rats with prior MCAo. (Reference-MR–positive refers to those pixels of the reference-MR image whose intensity exceeded 2 SD of left hemisphere values.) Data represent pooled pixel data from $n=3$ rats per group; pixels with ADC $>1.8 \times 10^{-3}$ mm$^2$/s were excluded because those values were assumed to represent cerebrospinal fluid–containing spaces. The ADC distribution of rats receiving albumin treatment is markedly rightward-shifted compared with the distribution of (reduced) ADC values seen in lesioned pixels of rats receiving saline ($P < 0.001$, Kolmogorov-Smirnov 2-sample test).

Figure 4. ADC distributions in reference-MR–negative pixels in saline- and albumin-treated rats with MCAo or sham occlusion. (Reference-MR–negative refers to those pixels of the reference-MR image whose values did not exceed 2 SD of mean left hemisphere values; $n=3$ rats per group.) The ADC distribution of albumin-treated rats is significantly rightward-shifted compared with saline-treated rats both in the reference-MR–negative pixels of the right (ipsilateral) hemisphere of rats with MCAo, as well as in the left hemisphere of both MCAo rats and sham-occluded animals ($P < 0.05$, Kolmogorov-Smirnov 2-sample test).
Nonetheless, comparisons revealed a close correspondence between the 2 data sets (Figures 2A, 2C, and 7).

To assess the frequency of selective ischemic neuronal changes without pannecrosis in a neocortical region in which infarction would invariably occur in the absence of albumin therapy but which was rescued by this therapy, we quantified the numbers of eosinophilic cortical neurons in the lateral cortex of albumin-treated rats \((n = 9)\). In 3 brains, this zone exhibited pannecrosis. In the remaining 6 brains, \(6.2 \pm 5.4\) (SD) necrotic (eosinophilic) neurons were present per \(3 \times 100\) microscopic field (range, 0 to 14). These neurons were typically located in small clusters within the middle cortical laminae.

**Brain Swelling**

Figure 8 depicts the rostrocaudal distribution of brain swelling in the 2 groups of the pooled series. Albumin administration strikingly reduced brain swelling at almost every coronal level studied and dramatically reduced the total percentage of brain swelling compared with saline-treated rats \((5.7 \pm 1.8\%\) and \(11.5 \pm 2.3\%,\) respectively; \(P = 0.00003\)).

**Endothelial and Microglial Alterations**

DVI observations indicated that albumin therapy not only reduced total lesion volume but also altered intracellular water within the lesion itself (Table 3, Figures 3 and 4). We thus wished to learn whether the morphological components of the ischemic infarct were themselves altered by albumin therapy. Histopathology revealed that the infarcted regions of saline-treated rats exhibited the typical microscopic features of subacute pannecrosis, with disappearance of both normal neurons and glia, markedly diminished numbers of identifiable microvessels, and vacuolar/rarefactive changes of the neuropil. By contrast, zones of infarction in albumin-treated rats showed better preservation of neuropil and numerous, readily identifiable microvessels with intact endothelium (Figure 9). A direct comparison of microvessel density within the central striatal infarct of albumin- versus saline-treated rats revealed moderate-to-increased microvessels in virtually all but 1 albumin-treated animal, but only sparse microvessel density in the majority of saline-treated animals. Lectin immunostains revealed sparse numbers of activated microglia within the infarcted central striatum of saline-treated rats but prominent numbers of ramified microglia within the central striatal infarct of albumin-treated rats (Figure 9). These observations are consistent with previous work showing that prominent microglial activation is a characteristic of mildly damaged, but not severely damaged, ischemic tissue. \(^{27}\)

<table>
<thead>
<tr>
<th>TABLE 4. Infarct Volumes and Brain Edema</th>
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<tr>
<td>Series 1 (MRI+histology) ((n=3))</td>
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<td>Infarct volume, (\text{mm}^3)</td>
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<td>Series 2 (histology only) ((n=4))</td>
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<td>Infarct volume, (\text{mm}^3)</td>
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<td>Striatum</td>
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<tr>
<td>Total</td>
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<td>% Brain edema</td>
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Data are mean±SEM.

\*Different from MCAo-Saline group \((P<0.05,\) Student’s \(t\) test).

Figure 5. Infarct areas (mean±SEM) in saline- and albumin-treated rats with MCAo followed by 3-day survival for light-microscopic histopathological quantification of infarction. There is a marked reduction in cortical infarct area (A) apparent at 7 coronal levels \((P<0.05,\) repeated-measures ANOVA with Bonferroni comparisons). The subcortical (caudoputamen) infarct area (B) is also significantly reduced at bregma level \(−1.3\) mm \((P<0.05,\) #\(P=0.05,\) repeated-measures ANOVA with Bonferroni comparisons). Total integrated infarct volume (C) is reduced by 66\% in albumin-treated rats \((P<0.05,\) Student’s \(t\) test). All data represent pooled values from series 1 and 2 (saline group, \(n=7;\) albumin group, \(n=9\)).
Discussion

This study was designed to assess the anti-ischemic efficacy of human serum albumin by evaluating 5 relevant end points: DWI, reference MRI, light-microscopic histopathology, planimetric measurement of brain swelling, and neurobehavior. By each of these criteria, albumin therapy instituted after a 2-hour period of MCAo proved to be strikingly efficacious. Thus, the neurological score of albumin-treated rats showed a sustained improvement averaging 43% to 50% over the 3-day survival period (Figure 1). In each of the 2 independently analyzed histological series that constituted this study, albumin treatment led, on average, to a 78% to 100% reduction of cortical infarct volume, a one-third reduction of striatal infarct volume, and a virtual elimination of brain swelling (Table 4). Central to our demonstration of albumin-associated neuroprotection were the following: (1) the use of a highly reproducible, physiologically regulated rat model of temporary focal ischemia, which we have thoroughly characterized in previous studies; and (2) the application of computer-assisted image-averaging strategies, which permitted pixel-based analysis.

Reference MRI 24 hours after MCAo revealed an albumin-associated reduction in lesion size that was confirmed by histopathology 2 days later (Figures 2 and 7). Additional insights were provided by the use of DWI, which measures the “self-diffusion” or random, brownian motion of water molecules among one another. The ADC is highly sensitive to parenchymal alterations produced by ischemia. Since Moseley and colleagues first reported regional hyperintensity and decreased ADC after experimental focal cerebral ischemia, these observations have been widely confirmed (eg, Reference). The MR diffusion signal has attracted particular interest in ischemic stroke because restricted diffusion is already apparent within only 5 to 30 minutes of onset. The concomitants of this early DWI hyperintensity and decline in ADC values include the following: (1) cytotoxic edema, ie, cellular ionic dyshomeostasis associated with failure of energy-requiring Na\(^+\)-K\(^+\)-ATPase pumps, leading to increases in intracellular Na\(^+\) and water, intracellular volume increase, and extracellular volume decrease; and (2) tissue acidosis and energy-metabolite depletion. In addition, ischemic depolarizations, which arise in the ischemic penumbra and contribute to penumbral deterioration and infarct growth, are associated with ADC decreases, whose recovery time exhibits a significant negative correlation with the degree of perfusion deficit. Finally, ADC values vary directly with brain temperature. Since brain temperature may decline during ischemia, this may confound the interpretation of altered ADC in ischemia. In the present study, however, temperature was controlled at normothermic levels.
The DWI data of this study show that albumin therapy not only substantially diminished the region of restricted diffusion after MCAo but, in addition, tended to normalize the ADC even within those pixels that, by reference-MR criteria, were ischae-

ically lesioned. This finding bespeaks a marked effect of albumin therapy in modifying cytotoxic edema within the ischemic focus, an effect supported by the virtual elimination of brain swelling (by planimetric criteria) in albumin-treated rats (Figure 8). Indeed, the antisywelling effect of albumin exceeded the magnitude of infarct volume reduction per se. This effect was reflected, as well, in systematic rightward shifts of ADC histo-

grams within unlesioned image pixels of the ipsilateral and contralateral hemispheres, in both rats with MCAo and sham-

operated controls (Figure 4).

It is possible that the amelioration of ADC decline ob-

served with albumin therapy is a consequence of enhanced regional perfusion of ischemic tissue. Quantitative blood flow studies in our laboratory, however, suggest that CBF aug-

mentation, while contributory, is not the sole mechanism of the efficacy of albumin.40 MR studies have shown that ischemia-induced decreases in ADC value are reversible with sufficiently prompt reperfusion.41,42 Other therapeutic strate-

gies, including hypothermia43 and pharmacological neuropro-
tectants (eg, References 44 and 45), are also capable of ameliorating or reversing DWI abnormalities.

Present-day echo-planar imaging methods permit repeated multislice DWI studies to be performed with great rapidity.46 In one study,46 the mean ADC value declined by 56% from control values of 0.92×10−3 mm²/s within 6 hours and remained decreased for 3 to 4 days, later “pseudonormalizing” at 5 to 10 days and becoming elevated chronically. In the present series, tissue regions showing ADC declines at 24 hours after MCAo coincided exactly with hyperintense re-

gions on reference MR images and (within the limitations imposed by slightly differing planes of histological section-

ing) with morphological infarcts in all cases. By 24 hours, zones of restricted diffusion correspond closely to the entire region destined for infarction.47,48 Mancuso et al,47 using both quantitative CBF and DWI in rats with 90-minute MCAo, demonstrated a correspondence between tissue regions hav-

ing reduced ADC values of ≥15% and zones in which CBF was reduced to 30% to 35% of normal. This CBF level, lying near the upper boundary of the ischemic penumbra,49 has been shown in our own recent quantitative studies to be at high risk of infarction after MCAo.50,51 Studies using both DWI and contrast-enhanced (“bolus track”) MRI to assess regional perfusion after MCAo have described prompt and significant ADC declines in core zones having the most compromised perfusion, but delayed and less pronounced ADC reductions in perifocal zones.52 There appears to be no single threshold of reduced ADC value capable of independ-

ently predicting irreversible injury, however, unless the duration of ischemia is also taken into account.53

MCAo reduced the mean ADC of the contralateral hemi-

sphere by ≈20% relative to the left hemisphere of sham-
occluded rats (Table 3). While we do not have a ready expla-
nation for this finding, bilateral (ie, transhemispheric) effects are well known after MCAo. For example, a contralateral “diaschi-

sis” of local cerebral blood flow and glucose metabolism has been well documented (see Reference 54 for review). These phenomena appear to have both a neural and possibly a neuro-
humoral basis. The present results are consistent with a bihemi-

spheric disturbance produced by unilateral MCAo.

The microscopic appearance of infarcted regions of albu-

min-treated brains differed from that of the saline-treated series in showing less prominent pannecrosis, persistence of vascular endothelium within the infarcted zone, and promi-

nent microglial activation. These findings suggest that albu-

min therapy may have important consequences beyond merely diminishing swelling and infarct volumes, viz, in preserving the neuropil within zones of residual infarction.

Several mechanisms by which albumin therapy may have induced neuroprotection in this study must be considered. These include hemodilution, oncotic effects, and rheologic mecha-
nisms. Albumin administration induced a prompt decline in hematocrit that recovered to normal by 24 hours (Table 1). In other studies, albumin treatment has also led to substantial hemodilution.\textsuperscript{8–10,55} Hemodilution may act by lowering blood viscosity and decreasing the aggregation of formed blood elements.\textsuperscript{7} Concentrated albumin solutions also have important oncotic effects, acting as a dehydrating agent to produce a net movement of water from tissue to blood. Cerebral swelling may thereby be prevented or significantly reduced.\textsuperscript{9,10} An advantage of albumin over the dextrans in this regard is the prolonged half-life of albumin in the circulation (\textsuperscript{7,56} 20 days).\textsuperscript{7,56} Because albumin molecules do not easily leave the circulatory system, they are capable of increasing plasma oncotic pressure over prolonged periods of time.\textsuperscript{57} Intravascular volume is normally regulated by the effective osmotic pressure of plasma proteins—the colloid oncotic pressure. Without this pressure, the hydrostatic pressure imposed by the heart rapidly drives plasma fluid into the interstitial space. The plasma protein that contributes most (80\%) to oncotic pressure is albumin.\textsuperscript{56} In our study albumin treatment given after 2-hour MCAo did not change plasma osmolality at 15 minutes or 24 hours but significantly increased plasma colloid oncotic pressure at 15 minutes. Similarly, plasma osmolality was not significantly affected by treatment with 25\% albumin in a study of cold injury,\textsuperscript{58} while colloid oncotic pressure was significantly higher in albumin- than in saline-treated animals after focal cerebral ischemia in gerbils.\textsuperscript{59}

In our study brain water content was elevated 24 hours after MCAo. Other studies have reported progressive increases of brain water content within 1 day after MCAo, followed by a gradual decline by 14 days.\textsuperscript{60} In a study of head injury in dogs, water content estimated by the wet weight/dry weight method was significantly decreased by multiple treatments with 25\% albumin (administered at 1 and 5 hours after the lesion).\textsuperscript{58} Similar reductions were reported when albumin was administered repeatedly to gerbils and rats with focal ischemia.\textsuperscript{10,59} By contrast, when a single injection of albumin was used, Clasen et al\textsuperscript{61} failed to show a reduction of water content after cold injury in dogs. In the present study we used a single albumin treatment at 2 hours after MCAo and were unable to show an effect on brain water content measured in ischemic tissue at 24 hours.

The benefit of albumin in this study, which was achieved without altering systemic blood pressure or other physiological variables, is consistent with the possibility that decreased blood viscosity may have been central to the therapeutic effect. It is unlikely, however, that the hemodiluting effect of albumin is solely responsible for its marked efficacy since numerous experimental and clinical trials of hemodilution

\begin{figure}
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\includegraphics[width=\textwidth]{figure9.png}
\caption{High-power microscopic fields (\texttimes400) showing infarcted region of central caudoputamen in a saline-treated (A, C) and albumin-treated (B, D) rat. A and B, Hematoxylin and eosin stain. The infarcted caudoputamen of the saline-treated rat (A) shows destruction of neuronal, glial, and vascular elements. By contrast, the albumin-treated brain (B) shows striking preservation of microvascular endothelium. C and D, Isolectin immunostain. Activated microglia are rare in the central striatal infarct of saline-treated rat (C), while abundant activated microglia with ramified processes are present in the infarct of albumin-treated rat (D).
}\end{figure}
with dextran or other agents have been negative or inconclusive, as noted previously in this report. It is possible that for hemodilution to be effective in the setting of acute stroke, it must be performed much earlier and to a more profound degree than was accomplished in previous studies.

It is possible that the specific physicochemical characteristics of albumin, and not merely its colligative properties, contributed to the therapeutic effect. For example, several reports strongly support a physiological role for human serum albumin as a scavenger of oxygen free radicals.6,56,62,63 The potential importance of this mechanism in ischemic injury is emphasized by the fact that albumin is present in relatively high concentrations in both plasma and interstitial fluid; hence, it is strategically situated to scavenge oxygen radicals and also to interrupt the damaging oxidative process of lipid peroxidation. Albumin can also bind copper ions, thereby inhibiting copper ion–dependent lipid peroxidation and hydroxyl radical formation.57 Wasil et al52 reported that albumin is also a powerful scavenger of hypochlorous acid in plasma and protects against H2O2–induced inactivation of α1-antiprotease. Finally, albumin can also bind free fatty acids and protect them from peroxidation.56 The increased vascular permeability secondary to blood-brain barrier breakdown in zones of focal ischemia may facilitate the antioxidant action of albumin by allowing increases in the extracellular fluid content of albumin to occur.56

Another action of albumin is its inhibitory effect on pathological platelet aggregation.64 This may be due to the fact that lysophosphatidic acid, the principal active serum phospholipid, is released from platelets during blood coagulation and binds tightly to albumin.65 In addition, albumin is an important plasma component responsible for inducing astrocytic proliferation.65 The normally tight blood-brain barrier prevents cells of the central nervous system from coming into contact with albumin and other protein components of the blood. Astrocytes and other glial cells proliferate to form glial scars when the blood-brain barrier is disrupted.65 Plasma albumin is a potent trigger of calcium signals and DNA synthesis in astrocytes.65 Stimulation of DNA synthesis is a normal precursor of mitosis, implying that albumin might act as a mitogen in astrocytes.

Albumin is distinguished from other colloids and crystalloids in its unique ability to bind reversibly with both anions and cations; hence, albumin can transport a number of substances, including fatty acids, hormones, enzymes, dyes, trace metals, and drugs.6 Substances that are toxic in the unbound or free state are generally not toxic when bound to albumin.

In conclusion, the present results provide encouraging support for the therapeutic administration of human serum albumin in the acute treatment of ischemic stroke. Our findings document that high-concentration albumin therapy instituted even 2 hours after the onset of temporary focal ischemia reduces infarct size, virtually abolishes brain swelling, and shifts parenchymal water homeostasis toward normal. Our data encourage the further development of this promising therapeutic strategy.

Acknowledgments

This study was supported by US Public Health Service grant NS 05820. The authors thank Susan Kraydieh for her expert technical assistance.

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Belayev et al previously presented very intriguing results concerning the neuroprotective effect of albumin against ischemia in a rat MCAo model. Now the authors are searching for the possible mechanism of this effect. MRI, especially diffusion MRI, is certainly one of the most suitable tools to investigate this question. Despite what could be considered unfavorable experimental conditions (1.5-T magnet, low gradient power, long echo time, and consequently low signal-to-noise ratio of ADC maps), the authors have succeeded in showing a striking result. Albumin, even at 24 hours after administration, increased ADC by 20% above its normal value in sham-treated rats or contralateral hemisphere and, more surprisingly, “normalized” ADC in the ipsilateral hemisphere. Correspondingly, the area of infarction also decreased dramatically. While this represents an important step toward understanding the effect of albumin, it seems that the result raises as many questions as it answers. This is partly because the mechanism of the ADC drop with ischemia is still not completely understood even 8 years after it was first documented by Moseley et al. Presently, the most popular hypothesis is that decreases in ADC reflect early cytotoxic edema (for review, see Reference 3). For example, assuming that ADC is lower in the intracellular space, a water shift from the extracellular to intracellular space should lead to an overall decrease in the measured diffusion constant. Increased tortuosity of water diffusion in the extracellular space in such conditions may be another possibility. However, Duong et al recently showed that the apparent water diffusion constants of the extracellular and intracellular space are almost equal before and after ischemia and that they both decrease with ischemia. This strongly suggests that the decrease in ADC is due to the decrease in energy-dependent cytoplasmic motion. In any event, it is widely accepted that cellular energy depletion is the dominant factor for ADC reduction due to ischemia. How does the “increase” in ADC observed in this study fit into this picture? What is the meaning of “normalized ADC”? Because the histology and T2 images show that a part of the albumin-treated brain with the normalized ADC is infarcted with MCAo, such regions are not really normal. This suggests that there may be a factor other than energy state that profoundly affects ADC values.

What does the increased ADC mean in terms of the protective effect of albumin against infarction? If the prevention were due to a very specific pharmacological effect, such as removal of a specific reactive chemical species, one would expect only a decreased infarction size and not a global ADC change. It would certainly be an interesting experiment to observe the time course of ADC change in its earlier stage of stroke. This would provide important information on the extent of the initial ischemic area during occlusion or immediately after reperfusion and on how much area is salvaged by reperfusion and/or albumin administration. The rate of recovery after reperfusion from the initial insult may offer a new clue into the mechanism of the albumin effect.

This landmark study will surely spur a surge of related investigations by those interested in stroke therapy, in the mechanism of stroke itself, and in the mechanism of ADC change in the brain.

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Diffusion-Weighted Magnetic Resonance Imaging Confirms Marked Neuroprotective Efficacy of Albumin Therapy in Focal Cerebral Ischemia

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Stroke. 1998;29:2587-2599
doi: 10.1161/01.STR.29.12.2587

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

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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