Albumin Therapy of Transient Focal Cerebral Ischemia
In Vivo Analysis of Dynamic Microvascular Responses

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Background and Purpose—To study whether intravascular or hemodynamic factors contribute to the marked neuroprotective effect of albumin therapy in focal cerebral ischemia, 2 complementary methods were applied: laser-scanning confocal microscopy (LSCM) and laser-Doppler perfusion imaging (LDPI).

Methods—In the LSCM study, Sprague-Dawley rats were anesthetized with halothane/nitrous oxide, and a cranial window was placed over the dorsolateral frontoparietal cortex. Rats received 2-hour middle cerebral artery occlusion (MCAO) by an intraluminal suture and were treated with human albumin (1.25 g/kg; n = 4) or saline (n = 3) after 30 minutes of recirculation. Video images of cortical vessels were continually acquired and were digitized offline to measure diameters and fluorescent erythrocyte velocities. In the LDPI study, cortical perfusion was measured in anesthetized Sprague-Dawley rats that received 2-hour MCAO and were treated with human albumin (2.5 g/kg; n = 6) or saline (n = 5) at 30 minutes after recirculation.

Results—In the LSCM study, MCAO was associated with arteriolar dilation and slowing of capillary and venular erythrocyte perfusion. During the first 15 to 30 minutes of posts ischemic recirculation, prominent foci of vascular stagnation developed within cortical venules, associated with thrombus-like foci and adherent corpuscular structures consistent in size with neutrophils. Saline administration failed to affect these phenomena, while albumin therapy was followed by significant increases in arteriolar diameter (∼12%; P = 0.007) and by a prompt improvement of venular and capillary erythrocyte perfusion and a partial disappearance of adherent thrombotic material. Albumin therapy increased erythrocyte flow velocity in both capillaries (288 ± 73% versus 76 ± 18% in the saline group; P = 0.023) and venules (2.7-fold [P = 0.001] versus 1.0-fold in the saline group [P = NS]). In the LDPI study, cortical perfusion declined during MCAO and rose initially with recirculation (to ∼135% of baseline) in both groups. Mean cortical perfusion improved slightly (∼14%; P = NS) in albumin-treated animals.

Conclusions—These results reveal a beneficial effect of albumin therapy in reversing stagnation, thrombosis, and corpuscular adherence within cortical venules in the reperfusion phase after focal ischemia and support its utility in the treatment of acute ischemic stroke. (Stroke. 2002;33:1077-1084.)

Key Words: albumin ■ middle cerebral artery occlusion ■ neuroprotection ■ perfusion ■ rats

We have recently shown that human serum albumin therapy confers marked neurological and histological protection in focal1–3 and global cerebral ischemia,4 as well as in traumatic brain injury in rats.5 The use of albumin solutions in the treatment of focal cerebral ischemia was initially prompted in part by the belief that hemodilution and reduction of blood viscosity would prove hemodynamically beneficial to ischemic tissue.6,7 Only a few previous studies have considered the effect of albumin treatment on cerebral perfusion after ischemia or related insults,7,8 and these studies have not provided definitive insights. In a recent autoradiographic study, we assessed local cerebral blood flow (CBF) at a single time point after albumin treatment of focal ischemia; we found that albumin therapy improved perfusion subtly within the former penumbra at 1 hour of recirculation after a 2-hour period of middle cerebral artery occlusion (MCAO). Because cerebral infarction is an evolving phenomenon, however, it is desirable to follow cerebral perfusion throughout the early recirculation period.

Albumin is known to have multifaceted intravascular effects. It exerts complex influences on erythrocyte aggregation, increasing low-shear viscosity but decreasing erythrocyte sedimentation under no-flow conditions.9 Albumin is also a specific inhibitor of endothelial cell apoptosis.10

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Severe albumin-binding proteins have been identified on endothelial cells from many organs, including brain, that mediate its transcytosis and endocytosis.\textsuperscript{11,12} Albumin also constitutes a major antioxidant defense against oxidizing agents generated both by endogenous processes (such as neutrophil myeloperoxidase) and by exogenous compounds.\textsuperscript{13,14} By binding copper ions, albumin inhibits copper ion–dependent lipid peroxidation and the formation of the highly reactive hydroxyl radical species.\textsuperscript{13,15} Albumin also plays crucial roles in the transport of fatty acids\textsuperscript{16,17} and in the binding of metabolites and drugs.\textsuperscript{18}

The recent introduction of laser-scanning confocal microscopy (LSCM) has permitted the real-time dynamic imaging of pial and cortical microcirculatory dynamics and the measurement of microvessel diameters and erythrocyte flow velocities in capillaries and venules.\textsuperscript{19} Another complementary method, laser-Doppler perfusion imaging (LDPI), measures relative parenchymal blood flow over a wide region of superficial cortical tissue within a short period of time by scanning a low-power laser beam across the brain, using the Doppler-shift signal to construct an image of relative blood flow within the measured region. (By contrast, conventional laser-Doppler flowmetry is restricted to a single point on the cortical surface.)

In the present study we used both LSCM and LDPI to explore whether hemodynamic alterations within the microvasculature are responsible for the marked neuroprotective effect of albumin in focal cerebral ischemia.

Materials and Methods

Laser-Scanning Confocal Microscopy

Animal Preparation
Male Sprague-Dawley rats (R. Janvier Breeding Center, France) weighing 274 to 326 g were studied after an overnight fast. Experiments were performed under permit No. 02934 from the French Ministry of Agriculture. After administration of atropine sulfate (0.5 mg/kg IP), animals were anesthetized with halothane (3.5% for induction, 1% for maintenance), 70% nitrous oxide, and a balance of oxygen; immobilized with pancuronium bromide (0.6 mg/kg IV); and mechanically ventilated. Rectal and cranial (left temporals muscle) temperatures were separately monitored with heating lamp and a feedback-controlled heating pad (Harvard Apparatus). Femoral artery and vein catheters were inserted to monitor arterial blood pressure (model RS3200 polygraph, Gould); to measure arterial blood gases (model 248, Ciba-Corning Diagnostics Corp), hematocrit, and plasma glucose (MediSense); and for infusion of drug and fluorescent tracers.

Middle Cerebral Artery Occlusion

The right common carotid artery was isolated from surrounding tissue through a midline incision, and the occipital branches of the carotid artery were coagulated. A 4-cm length of 3-0 monofilament nylon suture was inserted via the proximal external carotid artery were coagulated. A 4-cm length of 3-0 monofilament nylon suture was inserted via the proximal external carotid artery into the internal carotid artery, at a distance of 15 mm from the common carotid artery bifurcation. Before use, the suture was coated with poly-L-lysine solution as previously described\textsuperscript{20} to enhance its adhesion to the surrounding endothelium and increase the reproducibility of the resulting infarct.

The animal was then placed in a stereotactic head frame in the lateral position (David Kopf Instruments), and a midline skin incision approximately 1.5 cm in length was made parallel to the sagittal suture. To prepare the cranial window for LSCM, the cranial bone was thinned to translucency by means of a water-cooled dental drill over the right parietal cortex just behind the bregma. The thinned bone was carefully removed, and the dura mater was then lifted over an area of approximately 4 mm\textsuperscript{2}. Small incisions in the dura enabled it to be reflected at the cranial window periphery. A 150-μm-thick quartz microscope coverglass was cut to a size corresponding to the cranial window and was sealed to the bone by means of dental cement to make the preparation watertight. A right frontal craniotomy (diameter 2 mm) was also performed 2 mm rostral to the bregma and 2 mm lateral to the sagittal suture. The dura mater was left intact. An Ag/AgCl wire (diameter 0.07 mm, uncoated tip 1.5 mm) was placed between the calvarium and the meningeval surface to record the DC potential and the electrocorticogram. An Ag/AgCl disk electrode was inserted under the skin of the neck as a reference electrode.

The rat was then placed under the confocal microscope on a custom-built stereotactic device closely fitting the confocal microscope stage. Fluorescein-isothiocyanate (FITC)–dextran (molecular weight=70,000; 2.5 mg/mL) in 0.9% NaCl was injected (0.5 mL) intravenously to visualize microvessels and delineate their lumen. FITC-labeled erythrocytes (previously prepared in vitro\textsuperscript{21}) were injected via the same route at a tracer dose (<2%). LSCM was performed as previously described.\textsuperscript{19} A confocal laser-scanning unit (Viewscan, BioRad), attached to a microscope (Optiphoto-2, Nikon), was used. The scanning unit scanned 50 fields per second. The light source was an argon-krypton laser whose wavelengths were 488, 568, and 647 nm. After baseline images were collected, the middle cerebral artery (MCA) was occluded for 2 hours; the intraluminal suture was then carefully removed. Video images of cortical arterioles, capillaries, and venules were sequentially recorded before and during MCAO and for 2 hours of reperfusion.

Treatment

Human serum albumin (Alpha Therapeutic Corp, Los Angeles, Calif; 25% solution, 1.25 g/kg; n=4) or vehicle (sodium chloride 0.9%; n=3) was infused intravenously at a constant rate over 3 minutes, at 30 minutes after suture removal after a 2-hour period of MCAO.

Laser-Doppler Perfusion Imaging

Fasted Sprague-Dawley rats [weight, 276 to 337 g; Crl:CD (SD)BR strain, Charles River Laboratories, Wilmington, Mass] were anesthetized with 3.5% halothane in a mixture of 70% nitrous oxide and 30% oxygen. Animals were then paralyzed with pancuronium bromide, and mechanically ventilated. General surgical preparation and MCAO were the same as described above for the LSCM studies.

The animal was then placed in a stereotactic head frame in the lateral position (David Kopf Instruments), and a midline skin incision approximately 1.5 cm in length was made parallel to the sagittal suture and extended laterally to the midpoint between the right orbit and the external auditory canal. The temporalis muscle was left intact. An Ag/AgCl wire (diameter 0.07 mm, uncoated tip 1.5 mm) was inserted into the peritetrosquamosal suture, under direct visualization with a Zeiss surgical microscope. The MCA and its distal branches were clearly visible through the intact dura.

Cortical perfusion was measured with the Laser-Doppler Perfusion Imager (LDPI) (Moor Instruments). A computer-controlled optical scanner directed a low-power helium-neon laser beam over the exposed cortex. The scanner head was positioned parallel to the cerebral cortex at a distance of 26 cm. Each scan required 1.35 minutes for measurements of approximately 42×40 pixels (pixel size, 100 μm), covering an area of approximately 16 mm\textsuperscript{2}. At each measuring site, the beam illuminated the tissue to a depth of 0.5 mm. An image color coded to denote relative perfusion levels was displayed on a video monitor. After several baseline images were collected, the MCA was occluded for 2 hours, and the intraluminal suture was then carefully removed. Images were collected at 10-
Physiological Variables

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Physiological Variables

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MABP indicates mean arterial blood pressure. Values are mean±SEM.

*Different from saline group (P<0.05, Student’s t test).

Results

Physiological Variables

Albumin- and saline-treated animals of the respective series showed similar values for rectal and cranial temperatures, blood pressure, arterial blood gases, and plasma glucose (Table). Albumin therapy led to the expected moderate reduction in hematocrit (mean reduction, 28% to 29%; Table).

LSCM Study

Pial arterioles and venules under the closed cranial window were easily distinguishable from one another by the much more rapid flow of fluorescent erythrocytes through arterioles than through venules (Figure 1). Intraparenchymal capillaries were visible up to 200 μm beneath the surface of the brain by changing the focus of the microscope. They were characterized by a high tortuosity and by the heterogeneity of erythrocyte flow. MCAO was followed by arteriolar dilatation (Figure 1) with slowing of capillary and venular erythrocyte flow over 3 minutes, at 30 minutes after suture removal after a 2-hour period of MCAO.

minute intervals before and during MCAO and after initial reperfusion and at 5-minute intervals after treatment.

Treatments

Human serum albumin (25% solution, 2.5 g/kg; n=6) or vehicle (sodium chloride 0.9%; n=5) was infused intravenously at a constant rate over 3 minutes, at 30 minutes after suture removal after a 2-hour period of MCAO.

LDPI Analytical Strategy

In the course of MCAO and reperfusion, some degree of animal movement was inevitable. To preserve pixel-by-pixel alignment while tracking CBF changes by LDPI, we designed a new image-registration algorithm using an adaptive correlation approach to match the sequence of laser-Doppler images in the same spatial domain (P. Riyamongkol, MS, et al, unpublished data, 2001). In the first step, feature pixels were selected in a laser-Doppler baseline reference image. Local correlations were then performed between a “correlation window” of m×m pixels in the reference image and another window of n×n pixels (n>m) (termed the search window) in an image of the sequence to be registered. An iterative routine was used to obtain the alignment position yielding the maximum correlation coefficient. In the third step, image matching was performed by 2-dimensional polynomial transformation with bilinear interpolation. Once corresponding pixels were determined between images, each image in the sequence could then be mapped into the same spatial template as the reference image, and the whole image sequence could be automatically analyzed. Relative CBF data were then acquired by placing a polygon sample tool on each of the images, positioned 2 pixels away from vascular profiles.
perfusion. The occurrence of recurrent transient shifts in DC potential indicated peri-infarct depolarizations typical of the ischemic penumbra and confirmed the efficacy of MCAO. After removal of the intravascular suture following 2-hour MCAO, no increase in arteriolar blood flow was visible, and prominent vascular stasis developed within cortical venules, associated with thrombuslike stagnant foci (Figures 1 and 2) and adherent corpuscular structures (consistent in size with activated neutrophils) on venular endothelium (Figures 2 and 3). Administration of intravenous albumin after 30-minute recirculation was followed by a prompt improvement of venular and capillary erythrocyte perfusion and a partial disappearance of adherent intravenular material (Figures 1 and 2, bottom). In this animal, adherent material within venules persisted after saline administration Figure 2, middle), while subsequent albumin administration in this animal was followed by the prompt clearing of this adherent material (Figure 2, bottom).

**Quantitative Analysis**
Quantitative assessment of the cortical microvasculature was performed on videotaped images of cranial-window fields by an examiner blinded to the experimental conditions (J.S.). The diameter of arterioles (n=13) under basal conditions averaged 38.0±4.8 μm. Arteriolar diameters could be quantified sequentially during MCAO in 6 and 5 vessels of albumin- and saline-treated rats, respectively. For the pooled data, there were nonsignificant trends for arterioles to dilate (to 118±11% of their preischemic diameter) at the onset of MCAO and to recede (to 103±8% of preischemic diameter) by the end of MCAO (P=0.13, paired *t* test). After albumin administration, there was a highly significant increase in

**Figure 1.** In vivo fluorescent LSCM images of pial microvessels, rendered visible by FITC labeling (×20 objective). Before MCAO (Pre-MCAO), cortical venules (V) were clearly differentiable from arterioles (A) on the basis of their noticeably slower perfusion rates. During MCAO (MCAO, 37 minutes), dilation of cortical arterioles was noted (arrow). During the first half hour after the removal of the intravascular suture, before albumin therapy, a partial stagnation of venular reperfusion was observed, together with the striking appearance of thrombuslike material adherent to the venular endothelium (Recirc, 8 minutes and 26 minutes, arrowheads). By contrast, after administration of 1.25 g/kg of human albumin at 30 minutes of reperfusion, venular perfusion improved, and clearance of stagnant intravenular material was observed (Recirc, 49 minutes and 61 minutes).

**Figure 2.** Pial microvessels visualized by in vivo fluorescent LSCM with FITC-dextran–labeled plasma and FITC-labeled erythrocytes (×20 objective). Top, Before MCAO. Middle, After 2-hour MCAO and 38-minute postischemic recirculation (Recirc) (6 minutes after administration of 2 mL saline), thrombuslike materials together with nonfluorescent corpuscular structures, consistent in size with neutrophils, are adherent to venular endothelium (arrows). Bottom, At 123 minutes of postischemic recirculation, 4 minutes after the administration of albumin 2.5 g/kg, adherent material has diminished, and erythrocyte perfusion has improved. SAL indicates saline; ALB, albumin.

**Figure 3.** Pial venule before MCAO (left) and at 22 minutes of postischemic recirculation after 2-hour MCAO (right). After recirculation, multiple adherent nonfluorescent corpuscular structures are evident within the venule (arrows) (>20 objective).
mean arteriolar diameter of 11.6±2.8% over the ensuing 30 minutes (P=0.007, paired t test). By contrast, over the 30 minutes after saline treatment, mean arteriolar diameter decreased slightly (by 16±5%; P=NS, paired t test).

Erythrocyte flow velocity was measured in 11 cortical capillaries from 5 rats. Mean capillary flow velocity averaged 0.64±0.09 mm/s before MCAO and declined to 0.17±0.02 mm/s during early postischemic recirculation, before treatment. Thirty minutes after albumin treatment, capillary erythrocyte flow velocity increased by 288±73%, while 30 minutes after saline treatment, flow velocity was 76±18% of its value before therapy; this difference was highly significant (P=0.023; Figure 4).

Venular diameter estimated under basal conditions averaged 44.7±6.3 μm. Venular diameters were unaffected by MCAO, recirculation, or albumin or saline administration. Measurements of venular erythrocyte flow velocity made in the course of albumin or saline treatment are shown in Figure 4. Venular erythrocyte flow velocity failed to change significantly after saline treatment. By contrast, albumin treatment was followed by highly significant increases in venular erythrocyte flow velocity over the ensuing 30 minutes, amounting on average to 2.7-fold (Figure 4).

**Laser-Doppler Perfusion Imaging**

During MCAO, relative cortical perfusion declined to an identical extent in the 2 groups (saline group, 44.6±7.0% of preischemic baseline [mean±SEM]; albumin group, 44.6±5.3%; Figure 5). The initial increases in cortical perfusion during the first 30 minutes of recirculation after suture removal were also similar in the 2 groups (saline group, 139.5±23.2%; albumin group, 130.6±21.2%; Figure 5). After treatment (155 to 240 minutes; Figure 5), mean cortical perfusion tended to stabilize in the albumin group and to decline slightly in the saline group, but this difference was not statistically significant (mean values, 118.3±11.6% of baseline in the saline group and 135.2±10.6 in the albumin group; P=NS, repeated-measures ANOVA).

**Discussion**

LSCM and LDPI provide complementary information on the state of the cerebral circulation. While LSCM permits direct visualization of pial microvessels and allows the quantification of erythrocyte flow velocities within individual capillaries and venules, LDPI yields a relative measure of parenchymal blood flow in the superficial cortex. In the present study the salient (and unexpected) observation by LSCM related to prominent abnormalities developing within cortical venules of the ischemic penumbra in the early postischemic recirculation period, consisting of stagnation of flow, adherent thrombuslike material, and adherent corpuscular structures thought to be neutrophils. Administration of intravenous albumin after 30-minute recirculation tended to reverse these abnormalities, while treatment with saline was ineffective. LSCM quantification of erythrocyte flow velocities within capillaries and venules revealed increases after albumin (but not saline) treatment (Figure 4).

In contrast to the LSCM observations, relative cortical parenchymal flow as measured by LDPI was not significantly affected. The latter observation agrees with the results of our previous autoradiographic study of local CBF in this model: albumin-associated perfusion changes took the form of subtle CBF increases within a narrow circumferential zone of the former ischemic penumbra, which required the application of sophisticated computer-assisted image-processing methods for its detection. Within the LDPI region of interest (Figure 5), cortical perfusion declined to approximately 45% of control during MCAO—a level consistent with the ischemic penumbra. Since the coefficient of variation of this measurement was 32%, however, it is evident that the LDPI measurement polygon also included pixels lying within the ischemic core.

Taken together, our results suggest that, while albumin does not induce major increases in parenchymal perfusion, it nonetheless is highly effective in reversing stagnation and corpuscular adherence within the postcapillary microcirculation during the posts ischemic reperfusion period.
Dirmagl et al.\textsuperscript{23,24} introduced LSCM to study the cortical microcirculation of anesthetized rats via an implanted cranial window after the intravenous injection of fluorescein; a single line-scanning approach was used with 3-dimensional reconstruction, requiring several minutes for the acquisition of a 3-dimensional data set. Hudetz et al.\textsuperscript{25,26} developed an alternative method, using epifluorescence-intensified videomicroscopy in conjunction with FITC-labeled red cells to study the dynamics of the capillary microcirculation in the superficial cortex of anesthetized rats. LSCM used in conjunction with fluorescent plasma markers and 3-dimensional morphometry has also proven useful in studying fixed, stained serial brain sections derived from experimental insults.\textsuperscript{27,28}

The LSCM method used in the present study was first introduced by Seylaz et al.\textsuperscript{19} and became possible with the development of a 2-dimensional real-time confocal microscope permitting optical sectioning from the pial level to approximately 200 $\mu$m within the cortical parenchyma. The method allows rapid microcirculatory responses to brief forebrain ischemia to be visualized through a closed cranial window in anesthetized rats after fluorescent labeling of erythrocytes and plasma. Its advantages include its increased image resolution and improved depth of tissue penetration. Using this method, Pinard et al.\textsuperscript{29} characterized microvascular image resolution and improved depth of tissue penetration.

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**Figure 5.** Relative cortical perfusion (expressed as percentage of baseline) measured by LDPI in rats with 2-hour MCAO followed by recirculation; values are mean±SEM. Albumin or saline was administered at 30 minutes of recirculation (time ≈155 minutes). Representative computer-registered LDPI images are shown under baseline conditions, during MCAO, during early recirculation, and after albumin administration, respectively; polygons denote the region of interest from which the measurements were derived.

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Seeley et al.\textsuperscript{30} first demonstrated the progressive accumulation of polymorphonuclear leukocytes in regions of low CBF during early postischemic reperfusion. Several subsequent investigations have used intravital fluorescence videomicroscopy with rhodamine-labeled leukocytes to study leukocyte accumulation in focal and global ischemia.\textsuperscript{31,32} A recent study of 2-hour MCAO by the intraluminal filament method observed marked increases in leukocyte adhesion to venules at 15 to 60 minutes of recirculation (≈4.5-fold at 15 to 30 minutes, 25-fold at 1 hour), together with leukocyte rolling and significantly reduced shear rate in venules during the early reperfusion period.\textsuperscript{33} Other ischemia studies have confirmed that the preponderant locus of leukocyte adherence is to venular endothelium, which occurs during the first 3 hours of reperfusion and is suppressed by moderate hypothermia.\textsuperscript{31,34,35} By contrast, leukocyte adhesion within arterioles is less prominent.\textsuperscript{33,34} Transient MCAO with reperfusion appears to result in earlier neutrophil accumulation than permanent MCAO.\textsuperscript{36,37} When complete cerebral ischemia is of brief duration, accumulated leukocytes are dislodged from cerebral capillaries,\textsuperscript{38} while under conditions of persistently diminished cerebral perfusion, leukocytes may accumulate for several hours.\textsuperscript{39}

The adhesion of leukocytes to postcapillary venules has been identified as a critical first step in inflammatory processes affecting many organs, and strategies to impede neutrophil-endothelial adhesion and inhibit cellular injury have emerged as a promising therapeutic strategy in a variety of inflammatory states associated with ischemia/reperfusion, circulatory shock, and organ transplantation.\textsuperscript{40} Many studies in cerebral ischemia (especially in models of transient focal ischemia with reperfusion)\textsuperscript{17} have demonstrated the success of antileukocyte interventions in reducing infarct size and brain edema and in improving neurological dysfunction; recent reviews summarize many of these studies.\textsuperscript{41–43} Among the strategies shown in various models to antagonize leukocyte adherence and increased vascular permeability are glyc erol\textsuperscript{44} and hydroxethyl starch\textsuperscript{45}—agents that, like albumin, are osmotically active. By contrast, nitric oxide synthase inhibition augments and prolongs postischemic leukocyte adhesion by mechanisms thought to involve principally a decrease in velocity or shear rate,\textsuperscript{46} although others have suggested that nitric oxide exerts a chronic antiadherent effect in the cerebral microcirculation by inactivating adhesion-promoting superoxide radical formation.\textsuperscript{47} The latter observation is pertinent here in that serum albumin, via its free sulphydryl group, reacts with oxides of nitrogen to form a...
stable S-nitrosothiol with properties resembling endothelium-derived relaxing factor. By this means, albumin may both affect vascular tone and exert antiplatelet effects within the microcirculation.

In summary, the present findings suggest that a component of the neuroprotective effect of human albumin in acute ischemic stroke resides in its antagonism of stagnation, thrombosis, and leukocyte adhesion within the postcapillary microcirculation in the early reperfusion phase after focal ischemia, resulting in improved erythrocyte perfusion within the ischemic penumbra.

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