Prereperfusion Saline Infusion Into Ischemic Territory Reduces Inflammatory Injury After Transient Middle Cerebral Artery Occlusion in Rats

Yuchuan Ding, MD, PhD; Jie Li, MD; Josep A. Rafols, PhD; John W. Phillis, BVSc, PhD; Fernando G. Diaz, MD, PhD

Background and Purpose—In ischemic stroke, the ischemic crisis activates a cascade of events that are potentiated by reperfusion, eventually leading to cell death. The chief aim in this study was to investigate whether our new experimental model for stroke therapy, flushing the ischemic territory with saline before reperfusion, could minimize this damage by (1) reducing the inflammatory reaction and (2) improving regional microcirculation.

Methods—Stroke in Sprague-Dawley rats (n = 39) was induced by a 2-hour middle cerebral artery occlusion with the use of a novel intraluminal hollow filament. Before 48-hour reperfusion, 20 of the ischemic rats received 7 mL isotonic saline at 23°C or 37°C infused into the ischemic area through the filament. Regional cerebral blood flow in cortex supplied by the right middle cerebral artery was measured by laser-Doppler flowmetry during ischemia and reperfusion. Leukocyte infiltration, microvascular plugging, and infarct volume were compared with the use of hematoxylin and eosin staining. Expression of intercellular adhesion molecule 1 (ICAM-1) was determined by immunocytochemistry. Neurological deficits were evaluated.

Results—After the prereperfusion infusion of saline, significantly (P < 0.001) improved cerebral blood flow (105 ± 12% of baseline) was obtained up to 48 hours after reperfusion, compared with 45 ± 7% at 24 hours and 25 ± 3% at 48 hours after reperfusion without local saline infusion. Significant (P < 0.001) reductions in leukocyte infiltration (61%), vascular plugging (45%), infarct volume (approximately 65%), and neurological deficits were also produced. ICAM-1 expression in the infarct region was significantly (P < 0.05) minimized by 37%.

Conclusions—The reduced brain infarct and neurological deficits may be attributed to adequate reperfusion and ameliorated inflammation induced by local prereperfusion infusion. (Stroke. 2002;33:2492-2498.)

Key Words: cerebral ischemia infarcts intercellular adhesion molecule-1 leukocytes microcirculation reperfusion injury vascular plugging

Brain cells cannot survive under permanent severe ischemia, and early reestablishment of tissue perfusion would seem to be a logical first step in the treatment of acute ischemic stroke. However, reperfusion of ischemic brain, if it is not initiated early enough, can lead to extensive cell injury and death.1-3

There is abundant evidence that an acute inflammatory reaction associated with ischemia and reperfusion contributes to the development of cell injury in stroke.4-11 Cytokine production and molecular adhesive events that occur early in ischemia and the subsequent extensive recruitment of leukocytes to the ischemic zone during reperfusion lead to inflammatory injury. After the onset of ischemia, in addition to attracting leukocytes into ischemic sites, cytokines stimulate the synthesis of adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1), P-selectins, and E-selectins, on leukocytes, endothelial cells, and other cell types. Consequently, circulating neutrophils adhere to the endothelium, cross the vascular wall, and enter the brain parenchyma, followed by macrophages and monocytes. In addition, during and after occlusion of a larger upstream vessel, there is a tendency for the blood flow through microvessels to fall as a result of hemococoncentration, sludging of red cells, a disproportionate increase of viscosity, and platelet plugging of capillaries, with a reduced potential for substrate exchange.12-13

Mitigation of these events, therefore, should help to reduce the inflammatory reactions and establish an adequate perfusion of ischemic tissues, thus minimizing ischemia/reperfusion injury and potentially lengthening the therapeutic windows. To assess the feasibility of this concept, we flushed the vasculature of the ischemic territory with saline before the
onset of reperfusion using a novel intraluminal hollow filament. This new stroke model enabled us not only to occlude the middle cerebral artery (MCA) but also to administer solution through the hollow filament before establishing reperfusion. MCA intraluminal filament occlusion has been well accepted as an experimental stroke model in rats and mice since Koizumi and Nakazawa and Longa et al developed this technique 2 decades ago. This model mimics closely the clinical situation because the MCA is the most frequently embolized artery, and reperfusion occurs as recanalization is induced surgically or pharmacologically or as a result of spontaneous recanalization.

In a preliminary study, we demonstrated a significant infarct reduction and functional improvement up to 28 days after reperfusion in ischemic rats with the prereperfusion infusion. In this study we focused on whether our prereperfusion infusion procedure was effective in (1) improving the local microcirculation as determined by laser-Doppler flowmetry (LDF) and (2) minimizing the inflammatory response by interfering with interactions between reestablished blood flow with leukocyte influx and ischemically damaged tissue with expression of adhesion molecule protein.

Materials and Methods

Animals

Adult Sprague-Dawley rats (weight, 260 to 300 g; Charles River) were used and housed in the same animal care facility with food and water available during a 12-hour light/dark cycle throughout the protocol. Animal care and surgical procedures were performed in accordance with guidelines approved by the National Institutes of Health and the Wayne State University Animal Investigation Committee. In the control stroke group, 2-hour MCA occlusion was followed by 48 hours of reperfusion (n=10). In local infusion groups, after 2-hour MCA occlusion, a brief infusion of saline, at either body temperature (37°C) or room temperature (23°C), was conducted before onset of reperfusion to determine whether hypothermia also played a therapeutic role. Systemic infusion of the same amount of saline as used intracerebrally was applied in ischemic animals (n=9) to determine whether hemodilution was an issue in the study.

Induction of Stroke With a Hollow Intraluminal Filament

Animals were anesthetized and maintained with 1% to 3% halothane in 70% N₂O and 30% O₂, with a face mask. Rectal temperature was maintained at 37.5°C with a circulating heating pad and heating lamp throughout the surgical procedure. A length of 80 mm of PE-50 catheter was used, with one end of the catheter (18.5 to 19.0 mm) being modified to a filament with 0.2-mm outer diameter and 0.1-mm inner diameter. The filament was inserted into the right external carotid artery via an arteriotomy, while the right common carotid artery was temporarily clamped to reduce blood flow into the MCA-distributed area. After infusion, the catheter was completely withdrawn, and reperfusion was established. Blood pressure, blood gases (pH, Po₂, Pco₂), and hematocrit were measured through the right femoral artery before ischemia and 15 minutes after local or systemic saline infusion and followed by reperfusion in 12 ischemic rats. Brain temperature was measured with a YSI Telemetermeter 400 (YSI) in an additional 6 animals in the area supplied by the MCA in rats before, during, and after infusion at 0 through 10 minutes while blood flow was reestablished. Needle thermistor probes were placed into the cortex and striatum, through holes at 3 mm lateral to bregma and 3 mm posterior and 5 mm lateral to bregma, respectively.

Measurement of Local Cerebral Blood Flow

To verify MCA occlusion and reperfusion and to examine changes of the microcirculation after reperfusion, local cerebral blood flow (CBF) in cortex supplied by the right MCA was measured by LDF (Vasamedics Inc) before (baseline), during, and after (10 minutes, 24 and 48 hours) occlusion. A small craniectomy was made to expose the right parietal cortex. The LDF probe was stereotactically placed on the exposed, intact dura 3 mm posterior and 5 mm lateral to bregma. Since the size of the LDF probe was the same as the craniectomy hole, the probe could be placed exactly over the same cortical region for each measurement. Changes in regional CBF during ischemia and reperfusion were calculated as a percentage of the preischemic baseline. ANOVA was used to analyze statistical differences among groups, with significance level at P<0.05.

Neurological Examination

Neurological deficits in rats were examined during ischemia and reperfusion (at 20 minutes and 24 and 48 hours). The deficits were scored on a modified scoring system based on that developed by Longa et al as follows: 0, no deficits; 1, difficulty in fully extending the contralateral forelimb; 2, unable to extend the contralateral forelimb; 3, mild circling to the contralateral side; 4, severe circling; and 5, falling to the contralateral side. Neurological outcome in ischemic rats with local infusion was compared statistically with that of rats without the infusion treatment by ANOVA and simple mean effect analysis with a significance level at P<0.05.

Histological Analysis

Forty-eight hours after surgery, animals were deeply anesthetized with sodium pentobarbital (50 mg/kg IP) and killed by cardiac perfusion of saline followed by 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4. Four coronal brain slabs (each 2.0 mm in
thickness) were obtained with the use of a rat brain matrix (Activational Systems, Inc) from 4.0 mm (frontal cortex with sensorimotor cortex) to ~4.0 mm (middle of hippocampus) to the dura, as A, B, C, D.25 A and C slabs were processed by frozen sectioning to determine expression of ICAM-1 with immunohistochemistry, and B and D slabs were processed by paraffin sectioning stained with hematoxylin and eosin to determine leucocyte infiltration, vascular plugging, and infarct volume. The histological analyses were performed in a blinded fashion.

ICAM-1 Immunocytochemistry
Frozen sections cut coronally at a thickness of 30 μm were incubated with a monoclonal anti-rat ICAM-1 antibody (1:3000; IA29, Seikagaku) at 4°C for 48 hours. Expression of ICAM-1 was visualized by routine immunoperoxidase techniques.26 To determine the frequency of ICAM-1-positive vessels, the number of immunoreactive vessels <100 μm in diameter was counted and regarded as the total number of microvessels.26 Stained vessels were counted randomly throughout 8 lesion regions (2.5 mm² each) in the ipsilateral (ie, ischemic) hemisphere under a light microscope at ×400. Statistical differences in total numbers of labeled vessels in the counted lesion area (2.5 mm²×8≈20 mm²) between the 2 ischemic groups with or without prereperfusion infusion were analyzed by a 2-tailed Student’s t test with significance level at P<0.05.

Infiltration of Leucocytes and Vascular Plugging
To determine the numbers of polymorphonuclear leucocytes and vascular plugs in ischemic areas, 6-μm-thick paraffin sections stained with hematoxylin and eosin were prepared as described previously.28-29 From each ischemic hemisphere, 48 nonoverlapping microscopic fields (at a magnification of ×1000) were collected at 4 levels between frontal cortex (sensorimotor cortex) and middle of hippocampus, in 3 zones: cerebral cortex, striatum, and preoptic area. In each microscopic field, all leucocytes, including neutrophils and monocytes/macrophages, visible either in the vascular lumen and intramural wall or in the brain parenchyma, were counted. Leucocytes were identified by their nuclear features,30 and only cells with clearly identified nuclei were included in the counts. Total numbers of leucocytes observed and the average number per 1 mm² in the lesion area were calculated. Vascular plugs were counted also in the lesion site of those brains in which very few intravascular plugs were found in the contralateral hemisphere. Vascular plugs were defined as erythrocytes, leucocytes, and platelet fibrin thrombi visible inside capillaries (<15 μm). The magnitude of vascular plugging was presented as the percentage of the plugged vessels in each lesion region. Statistical differences in cell numbers, as well as in vascular plugs from the different ischemia groups with or without local infusion, were analyzed by a 2-tailed Student’s t test with significance level at P<0.05.

Infarct Volume
The infarct region, defined as the area with reduced staining or containing eosinophilic-necrotic cell bodies, was also determined in the sets of 4 serially cut sections through the MCA territory in the frontoparietal sensorimotor cortex and the dorsolateral aspect of the neostriatum. The infarct area was traced with a Tri Simplex projector (×15). By using a scanner and an image analysis system (Meta-Morph Imaging System, Universal Imaging Corporation), the areas of noninfarcted tissue ipsilateral and contralateral to the occluded side were measured. To minimize the error introduced by edema, an indirect method for calculating infarct volume was used.24,31 The noninfarcted region in the ipsilateral hemisphere was subtracted from that in the contralateral hemisphere. The infarct volume is presented as a percentage of the volume of the contralateral hemisphere. Statistical differences in infarct volume from different ischemia groups were analyzed by ANOVA and Duncan’s multiple range tests, with significance level at P<0.05.

Results

Neurological Examination
MCA occlusion in 4 groups (stroke, stroke with systemic infusion, stroke with local infusion of saline at 37°C, and stroke with local infusion of saline at 23°C) was examined at 4 time points during ischemia and reperfusion and scored on a 6-point scale (Figure 2). ANOVA with repeated measures on the time points indicated significant differences between the groups (F9,66=4.84, P<0.001), time points (F3,66=181.72, P<0.001), and interaction between the groups and time points (F9,66=3.08, P<0.001). Simple mean effect analyses were conducted and revealed that there were no differences among the 4 groups during ischemia and early reperfusion (20 minutes), but group differences were significant at 24 and 48 hours after reperfusion. Specifically, compared with the stroke and stroke with systemic infusion groups, neurological deficits in rats with local infusion of saline at 37°C were significantly (P<0.001) reduced from 24 through 48 hours. Infarction at 23°C further reduced the neurological deficits, although the observed changes did not reach a significance level.

Infarct Volume and Neuronal Damage
The infarct volume in ischemic brains with a 2-hour MCA occlusion followed by 48 hours of reperfusion was measured in the 4 stroke groups (Figure 3). Infarct within the territory of the occluded MCA included the frontoparietal cortex and the neostriatum. The boundaries between areas of infarct and adjacent normal brain were clearly delineated. ANOVA detected significant differences in infarct volume reduction (F3,35=58.77, P<0.001). Furthermore, with Duncan’s multiple range tests, the difference was revealed only between ischemic rats with and without a local infusion. No differences were detected between stroke (48±2%) and stroke with systemic infusion (44±3%) or between local infusion of saline at room (23°C) (13±2%) and body (37°C) (18±3%) temperatures, although the room temperature saline infusion further reduced infarct volume by 27% compared with body temperature saline. Thus, our data indicated a significantly reduced infarct volume (approximately 65%) in ischemic rats with a local saline infusion compared with that in rats without the infusion.
C and 37°C between animals with local infusion of saline at 23°C. Between stroke and stroke with systemic infusion animals or with and without a local infusion. No differences were detected by Duncan's multiple range tests, only between ischemic rats in rats without the infusion. The difference was revealed, either at body (37°C) or room (23°C) temperature compared with saline at 23°C. A significantly (*P<0.001) reduced infarct volume was found in ischemic rats with a local saline infusion systemically or locally (Table 1). Hematocrit differences and interactions between groups and time points during ischemia and early reperfusion (10 minutes) in the 4 ischemic groups: rats without or with systemic saline infusion and rats with local infusion at body or room temperature. Rather, it suggested that the degree of decrease in cortical perfusion (mean, 17% to 27% of preischemic CBF; SE, ±4% to 7%) was statistically comparable. Local CBF in these animals consistently recovered to preischemic levels by 10 minutes after onset of reperfusion (79±10%, 112±21%, 102±21%, 99±13% of preischemic CBF, respectively).

Furthermore, changes in local CBF as a percentage of the preischemic baseline during occlusion and up to 48 hours of reperfusion were compared between ischemic animals and ischemic animals with saline infusion at body temperature (Figure 4). ANOVA at 4 time point measurements revealed significant differences on groups (F(3,36)=7.29, P<0.05), time points (F(3,36)=16.56, P<0.001), and interaction between groups and time points (F(3,36)=7.93, P<0.001). An additional simple mean effect analysis indicated that group differences occurred only at 24 and 48 hours after reperfusion rather than during ischemia and early reperfusion. Specifically, CBF was reduced significantly (P<0.001) to 45±7% at 24 hours and 41±1% at 48 hours after reperfusion.

### TABLE 1. Physiological Variables in Ischemic Animals With Systemic or Local Saline Infusion

<table>
<thead>
<tr>
<th>Blood Pressure</th>
<th>pH</th>
<th>$\text{P}_{O_2}$</th>
<th>$\text{P}_{O_2}$</th>
<th>Hematocrit</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Stroke with systemic infusion</td>
<td>106±2</td>
<td>102±2</td>
<td>7.36±0.02</td>
<td>7.33±0.02</td>
</tr>
<tr>
<td>Stroke with local infusion</td>
<td>108±3</td>
<td>106±3</td>
<td>7.36±0.01</td>
<td>7.35±0.02</td>
</tr>
</tbody>
</table>

Before indicates before ischemia; After, 10 minutes after saline infusion followed by reperfusion. Values are mean±SE. No significances were detected by ANOVA analysis.
25±3% of preischemic level at 48 hours after reperfusion in the noninfused ischemic animals. In contrast, a relatively normal perfusion (105±12%) was obtained in the ischemic rats with local saline infusion.

Effort was made to ensure that the baseline was not changed by repeated CBF recording. We found only 5±1% of changes (n=12) by removing and reinserting the probe at 2 different times in the same animals.

**Inflammatory Events and Vascular Plugging**

Inflammatory events and vascular plugging were compared between the 2 ischemic groups with or without body temperature saline infusion 48 hours after ischemia and reperfusion (Table 2).

Expression of ICAM-1 was demonstrated by immunohistochemistry. Microvessels in the ischemic lesion in noninfused ischemic rats strongly expressed ICAM-1 immunoreactivity compared with that in saline-infused rats. Further quantitative analysis indicated that the number of ICAM-1–positive vessels in the prereperfusion infarction group was significantly \((P<0.05)\) reduced by 37%, from 104±8 to 66±5, per 20 mm\(^2\) of lesion region. Immunoreactivity was rarely found in brain tissue beyond infarct regions.

When the numbers of leukocytes in preoptic, striatal, and cortical lesions were compared, a 61% reduction was associated with prereperfusion infusion, with the total number of infiltrated leukocytes being significantly \((P<0.001)\) reduced from 720±51 to 282±40. The average number of leukocytes per 1 mm\(^2\) infiltrated into infarcted regions was also compared to ensure that the reduced leukocyte infiltration was not due to reduced infarct. A significant \((P<0.01)\) difference was also observed between noninfused (30±2/mm\(^2\)) and infused (15±2/mm\(^2\)) groups.

In the infarct region, the percentage of plugged vessels was significantly \((P<0.001)\) reduced by 45%, from 81±3% in ischemic animals without local infusion to 45±2% with local infusion.

**Discussion**

In the present study the therapeutic value of “flushing” the microvasculature in the ischemic territory before reperfusion (ie, prereperfusion infusion) demonstrated by the significant reduction (approximately 65%) in infarct volume was correlated with 61% reduction of leukocyte infiltration \((P<0.001)\), 37% reduction of ICAM-1 microvascular expression \((P<0.05)\), and 45% reduction of microvascular plugging \((P<0.001)\).

No significant differences in infarct volume were detected between ischemic animals without local infusion and those with a systemic saline infusion. In addition, we found no significant differences in physiological variables, such as arterial blood pressure, blood pH, blood PO\(_2\) and PCO\(_2\), and hematocrit, between the 2 ischemic rat groups before and after local or systemic saline infusion. These results suggest that the minor hemodilution after the local infusion via the MCA did not contribute to reducing brain injury. It is likely that the major therapeutic potential of the prereperfusion flushing might have been a consequence of local clearance and protection of ischemic tissue and that moderation in the reperfusion pace might also have minimized the reperfusion injury. In addition, we did not observe obvious light microscopic evidence of tissue and vascular alterations beyond the infarct area. These histological and physiological observations together suggest that local flushing did not induce brain tissue damage and that possibly increased local intraluminal pressure and blood volume from saline infusion did not produce forced dilatation of cerebral vessels and sustained elevations in CBF, as well as physiological changes.

The hollow filament used in experimental rats was not inserted directly into the MCA because of the small size of the vessel. Therefore, the anterior cerebral artery was not blocked during saline infusion. Although 7 mL is a relatively large amount of volume, it is likely that only half of the total solution, injected posterior to the junction of MCA and anterior cerebral artery, flowed into the MCA territory. This volume represents approximately 15% to 20% of the total blood volume in rats.\(^{32}\)

Brief postischemic mild cooling in the brain region supplied by the MCA resulted quickly from local infusion of saline at room temperature. Further reductions in infarct volume and neurological deficits, however, were not statistically significant compared with those seen after infusion with body temperature saline. Although it is unlikely that the observed brain cooling played a major role in reducing brain injury in this study, we cannot rule out a neuroprotective effect induced by hypothermia.

Cerebral infarction after MCA occlusion may be mediated by many factors, among which are inflammatory processes.\(^{4,11,26,27}\) Initially, ischemia triggers the expression of a number of cytokines, which attract leukocytes into ischemic sites and stimulate the synthesis of adhesion molecules such as ICAM-1 on migrated leukocytes, endothelial cells, and other cell types. The upregulation of these inflammatory mediators occurring during ischemia promotes recruited blood-borne inflammatory cell adherence and infiltration during reperfusion. Consequently, postischemic leukocytes exacerbate brain injury by physically obstructing capillaries.

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**Table 2. Inflammatory Events and Vascular Plugging in Infarcts of Stroked Brain**

<table>
<thead>
<tr>
<th></th>
<th>ICAM-1–Labeled Vessels, 20 mm(^2)</th>
<th>Total Number</th>
<th>Number per mm(^2)</th>
<th>Vascular Plugging, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stroke</td>
<td>104±8</td>
<td>720±51</td>
<td>30±2</td>
<td>82±3</td>
</tr>
<tr>
<td>Stroke with local infusion</td>
<td>66±5*</td>
<td>282±40†</td>
<td>15±2‡</td>
<td>45±2§</td>
</tr>
</tbody>
</table>

*\(P<0.05\), †\(P<0.001\), ‡\(P<0.01\), §\(P<0.001\), compared with no local MCA infusion of saline.
to reduce blood flow during reperfusion and/or by releasing cytotoxic products once migrated into the brain parenchyma. With the use of our hollow filament model, the inflammatory process could have been mitigated by removal of cytokines or/and inhibition of adhesion molecules. Whereas the reduction of infarct in this study is probably dependent on multiple factors, it is at least partially due to the decreased inflammatory reaction.

LDF is widely used for assessing local circulation because it provides real-time, noninvasive, and continuous semiquanitative data on cortical CBF.3–36 Our evidence of dynamic changes determined by LDF in the microcirculation during ischemia and reperfusion supports previous reports in rats12 and mice14 that a brief recovery of blood flow after MCA occlusion is followed by a significant hyperperfusion (20% to 60% of preischemic value). The decreased blood flow and postischemic hyperperfusion in the microcirculation were significantly improved by prereperfusion infusion in this investigation. Such infusion could have provided a local clearance and neutralization of mechanical and rheological events that occur in the microcirculation after occlusion of upstream arteries.1,12,13 Our results support the hypothesis of Olsson and Hossmann,37 which is based on intracarotid saline perfusion after transient global ischemia in cats 3 decades ago. They suggested that elimination of metabolic waste products and an improved postischemic circulation provided the mechanisms of neuroprotection. Similarly, the improved cerebral microcirculation in this study may have played a crucial role in infarct reduction.

In conclusion, flushing the MCA territory via a hollow filament minimizes ischemia/reperfusion injury in our stroke model. The therapeutic effect was related to a reduction of inflammatory events and improvement of the cerebral microcirculation. Our model could lead to development of novel therapy that combines local infusion of ischemic region with subsequent or coincident mechanical recanalization of the occluded artery and/or treatment of pharmacological agents.

Acknowledgments
This work was supported in part by a Wayne State University Research Stimulation Fund to Dr Ding. We want to thank Drs R-L. Zhang and K-F. Liu, Henry Ford Hospital, Detroit, Mich, for providing detailed surgical information on the intraluminal filament stroke model, as well as the histological techniques on inflammation. We are grateful to Dr Qin Lai for his help in the statistical analysis and to Dr Hun Park for his help in the arterial blood pressure measurements. Dr James P. McAllister is also appreciated for his support and encouragement of this study.

References


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Stroke. 2002;33:2492-2498
doi: 10.1161/01.STR.0000028237.15541.CC
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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