Effects of Retrograde Perfusion of the Brain With Combined Drug Therapy After Focal Ischemia in Rat Brain

M. Shimauchi, MD, and Y.L. Yamamoto, MD, PhD

Background and Purpose: The ischemic edema associated with blood–brain barrier permeability changes and the excess production of free radicals are serious complications in prolonged cerebral ischemia. We examined the efficacy of transvenous perfusion of the brain, starting treatment 5 hours after occlusion of the middle cerebral artery for a period of 2 hours in rats with the combined agents mannitol (10 ml/2 hr) and dexamethasone (1 mg/2 hr) to counter edema and verapamil (0.05 nig/kg/2 hr) for vasodilation.

Methods: In experiment 1, blood–brain barrier permeability changes were examined in five groups with six rats each: group C rats underwent 7 hours of middle cerebral artery occlusion with no treatment; group V, treatment with verapamil alone; group VD, treatment with verapamil and dexamethasone; group VM, treatment with verapamil and mannitol; and group VDM, treatment with verapamil, dexamethasone, and mannitol. In experiment 2, we examined local cerebral blood flow, ischemic tissue damage volume, and water content of cerebral hemispheres in two groups of 16 rats each subjected to the same treatment as groups C and VDM rats in experiment 1.

Results: There was a significant reduction of blood–brain barrier permeability changes in the ischemic cortex of rats in group VDM compared with rats in the other groups. In the group undergoing transvenous perfusion of the brain with the three combined agents, there was a significant improvement of cerebral blood flow (39–58%, p<0.05) in the ischemic cortex and reduction of ischemic cerebral damage volume (22%, p<0.01) and water content of the ischemic hemisphere (p<0.05) compared with the control group.

Conclusions: The therapeutic approach using combined agents is effective treatment when initiated within 5 hours of focal cerebral ischemia in rats. (Stroke 1992;23:1805–1811)

KEY WORDS • brain edema • cerebral ischemia • free radicals • rats

Experimental studies of cerebral ischemia have shown that some cytoprotective agents have limited efficacy in the early treatment of cerebral ischemia.1–8 However, if cerebral artery occlusion continues for a few hours, complications such as brain edema occur, and it is no longer easy to protect ischemic brain effectively. Transvenous retrograde perfusion of the brain (TVPOB) is a new approach in the treatment of acute ischemia.9 Our recent studies10 showed that TVPOB with verapamil had a beneficial effect on ischemic brain when treatment was started in rats 3 hours after middle cerebral artery (MCA) occlusion for a period of 2 hours; however, a favorable result was not obtained when this treatment was started over 5 hours after MCA occlusion, which resulted in serious complications such as brain edema and blood–brain barrier (BBB) permeability changes.

In long-term ischemia (over 5 hours), the problem of brain edema due to free radicals and BBB permeability changes has been indicated in the literature.3–12 In the present study, we first examined the effect of TVPOB with various combinations of the cytoprotective agents mannitol, dexamethasone, and verapamil on BBB permeability changes 7 hours after MCA occlusion in rats. We examined also the efficacy of TVPOB with combinations of the three agents on local cerebral blood flow (LCBF), ischemic cerebral damage volume, and ischemic brain edema in the same model.

Materials and Methods

Sixty-two Sprague-Dawley rats weighing 350–450 g were used for two experiments. In experiment 1, we used 30 rats divided into five groups of six rats each for evaluation of BBB permeability changes in focal cerebral ischemia by autoradiography using the 14C-labeled-α-aminooisobutyric acid ([14C]AIB) method developed by Blasberg et al.14 Focal cerebral ischemia in all rats was achieved through occlusion of the left MCA.15 Group C (control) rats underwent surgical preparations for MCA occlusion but received no cytoprotective agent treatment. Rats in the other four groups underwent cannulation of a cerebral vein16 in addition to occlusion of the left MCA and treatment with various combinations of cytoprotec-
tive agents. Group V rats received through TVPOB a constant infusion of 0.4 μg·kg⁻¹·min⁻¹ verapamil with 0.9% saline solution by IVAC infusion pump (IVAC Corp., San Diego, Calif.) for a period of 2 hours. Group VD rats received through TVPOB a combined treatment of the same dose (0.4 μg·kg⁻¹·min⁻¹) verapamil plus 20 μg·kg⁻¹·min⁻¹ dexamethasone for 2 hours by infusion pump. Group VM rats received through TVPOB a combined treatment of the same dose (0.4 μg·kg⁻¹·min⁻¹) verapamil, 20 μg·kg⁻¹·min⁻¹ dexamethasone, and 83 μl/min 20% mannitol for 2 hours by infusion pump. Group VDM rats received through TVPOB a combined treatment of the same dose (0.4 μg·kg⁻¹·min⁻¹) verapamil, 20 μg·kg⁻¹·min⁻¹ dexamethasone, and 83 μl/min 20% mannitol for 2 hours by infusion pump.

In experiment 2, we used 32 rats divided into two groups of 16 rats each. Rats in these two groups (A and B) were subjected to the same treatment as those in group C (control) and group VDM (TVPOB with the cytoprotective agents) was started 5 hours after MCA occlusion and continued for 2 hours. Arterial blood samples (50 μl) were then decapitated at 7 hours after MCA occlusion, and the brains were removed and immediately frozen. Each brain was sliced into 20-μm sections in a cryostat (−22°C), and each section was mounted on a slideglass and rapidly dried on a hot plate for autoradiography. Autoradiograms were made by 3-weeks' exposure of brain sections to Kodak SB-5 film (Rochester, N.Y.) with ¹⁴C standards. The optical density was measured in each of three consecutive autoradiograms and the mean value of the tissue ¹⁴C radioactivities was obtained for each locus. The densitometric measurements were made with a digital analyzer (The Image Calculator, McGill University, Montreal, Canada). The radioactivity of ¹⁴C in plasma was measured with a Model 1219 Rackbeta liquid scintillation counter (Wallac Oy, Turku, Finland). The Ks for each locus were calculated from the tissue ¹⁴C concentration obtained from the autoradiograms and the arterial plasma ¹⁴C concentration–time integral.¹⁴

The measurement of LCBF was made with ¹⁴C]IAP at 6 hours and 59 minutes after MCA occlusion. To measure LCBF, 30 μCi [¹⁴C]IAP was infused intravenously at a constant rate for a period of 1 minute. Arterial samples (20 μl) were drawn at 5-second intervals after the start of injection of [¹⁴C]IAP. The rats were then decapitated at 7 hours after MCA occlusion, and the brain sections for measurement of LCBF were obtained by an autoradiographic method similar to that for Ks measurement. Autoradiograms were made by 1-week exposure to the film with ¹⁴C standards. The tissue and whole blood ¹⁴C activities were measured, and LCBF was calculated with the operational equation described by Sakurada et al.¹⁶

Volume of ischemic cerebral damage was evaluated by a modification of Osborne's method.¹⁷ Twenty micrometers of seven coronal frozen tissue sections adjacent to those used for [¹⁴C]IAP autoradiograms were obtained at 1.28-mm intervals from the level of 3.16 mm anterior to the occipital tip. These freeze-dried sections were then fixed in 10% formaldehyde solution for over 24 hours and stained with cresyl violet and Luxol fast blue. These staining sections resulted in well-demarcated ischemic cerebral damage borders that were readily detected by a digital image analyzer system (The Image Calculator). Any indistinct border of ischemic cerebral damage was verified under light microscopic examination for neuronal ischemic damage. This freeze-dried sectioning method resulted in an advantage of simultaneous parallel investigation of multitracer autoradiography for quantitative measurement of pathophysiologic parameters and histopathologic investigation in the same ischemic tissue. Areas of ischemic cerebral damage were measured on seven equally spaced sections determined by anatomical landmark from the atlas of Paxinos and Watson.¹⁸ The total volume of ischemic cerebral damage was calculated by a computer program that summed all sectional areas multiplied by the interval distance (1.28 mm). The volumes of the ischemic and nonischemic hemispheres were also measured.
Brain edema was evaluated by the brain edema index (BEI) and water content of the cerebral hemispheres. Brain edema index was expressed as a ratio of the difference between the volume of the ischemic (left) hemisphere and the nonischemic (right) hemisphere to the total cerebral volume as follows:

\[ \text{BEI} = \frac{\text{Volume of left hemisphere} \ - \ \text{Volume of right hemisphere}}{\text{Total cerebral volume}} \]

To measure water content, the rats' brains were removed as soon as possible 7 hours after MCA occlusion. Brains were divided as to hemisphere, placed in preweighed glass vials, weighed, and dried for 6 days at 90°C. The percentage of water in the cerebral hemisphere was calculated using the formula \( \text{BEI} = \left( \frac{\text{wt} - \text{dry wt}}{\text{wt} \times 100} \right) \times 100 \).

All data were expressed as mean±SEM. The statistical analysis of all data was performed using one-way analysis of variance (ANOVA), followed by Tukey's test. A value of \( p<0.05 \) was considered significant.

**Results**

No significant intergroup differences were noted in arterial blood pressure, blood gases, hematocrit, or blood glucose in the five groups during the experiments.

The values of BBB permeability (\( K_v \)) are summarized in Table 1 and Figure 1. \( K_v \) values were significantly increased 50-60% (\( p<0.01 \)) in the ischemic cortical areas compared with the homologous areas in the nonischemic hemispheres in groups C, V, VD, and VM (Table 1), indicating significant alteration of BBB permeability changes observed in the ischemic hemispheres of these groups. In group V, \( K_v \) values in the ischemic cortical areas were further increased compared with those in the control group, indicating worsening of BBB permeability due to the vasodilating effect of verapamil. Statistical analyses using ANOVA with Tukey's test revealed that there were significant differences of \( K_v \) values in the ischemic frontal, sensorimotor, and anterior parietal cortical areas between groups V and VDM (Figure 1). There was also a significant difference in the ischemic frontal cortex between groups V and VD, and in the ischemic sensorimotor cortex between groups V and VM, indicating that BBB permeability was further aggravated by TVPOB with verapamil alone in the ischemic cortical areas but that there was a significant improvement in BBB permeability change with the addition of either 20% mannitol (group VM) or high-dose dexamethasone (group VD) combinations.
Table 2. Effect of TVPOB With Combined Cytoprotective Agents on Local Cerebral Blood Flow After 7 Hours of Left Middle Cerebral Artery Occlusion

<table>
<thead>
<tr>
<th>Area</th>
<th>Control (n=10)</th>
<th>TVPOB (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
<td>R</td>
</tr>
<tr>
<td>Cingulate cortex</td>
<td>113±8</td>
<td>149±8</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>51±5</td>
<td>144±9</td>
</tr>
<tr>
<td>Sensorimotor cortex</td>
<td>36±4</td>
<td>151±9</td>
</tr>
<tr>
<td>Anterior parietal cortex</td>
<td>38±4</td>
<td>165±8</td>
</tr>
<tr>
<td>Posterior parietal cortex</td>
<td>41±4</td>
<td>169±9</td>
</tr>
<tr>
<td>Auditory cortex</td>
<td>65±7</td>
<td>174±9</td>
</tr>
<tr>
<td>Occipital cortex</td>
<td>75±6</td>
<td>153±9</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>83±5</td>
<td>100±8</td>
</tr>
<tr>
<td>Amygdala</td>
<td>64±11</td>
<td>94±6</td>
</tr>
<tr>
<td>Caudate lateral</td>
<td>4±1</td>
<td>146±8</td>
</tr>
<tr>
<td>Caudate medial</td>
<td>78±7</td>
<td>121±8</td>
</tr>
<tr>
<td>Posterolateral portion of CP</td>
<td>74±13</td>
<td>135±8</td>
</tr>
<tr>
<td>Globus pallidus</td>
<td>69±5</td>
<td>76±5</td>
</tr>
</tbody>
</table>

Values are mean±SEM and given as milliliters per 100 grams per minute. TVPOB, transvenous perfusion of the brain. *p<0.05 significantly different from control group by one-way analysis of variance.

Compared with verapamil alone (group V). However, there was no significant difference between groups C and VM or VD. There were no significant changes of Kt values in the ischemic cortical areas compared with the homologous areas of the nonischemic hemispheres in group VDM, indicating a significant improvement in or protection of BBB permeability changes by TVPOB with the combined treatment of verapamil, dexamethasone, and 20% mannitol. The values of Kt in regions of nonischemic cerebral hemisphere did not differ among the five groups.

Local cerebral blood flow in the two groups is summarized in Table 2 and Figure 2. In group B (TVPOB), there was a significant improvement of LCBF in the left sensorimotor (58%, p<0.05) and parietal cortices (39–63%, p<0.05) in the ischemic hemisphere compared with that of group A (control) by ANOVA with Tukey's test. Quantitative analyses of both size and volume of ischemic cerebral damage indicated that group B (TVPOB) rats showed a significant reduction of both size and volume of ischemic cerebral damage compared with those in group A (Figure 3). There was a significant reduction in size of ischemic cerebral damage at the sensorimotor (30%, p<0.05) and the anterior parietal levels (19%, p<0.05) in the group B rats compared with those in group A by ANOVA with Tukey's test. The total volume of ischemic cerebral damage was significantly reduced by 22% (p<0.01) in group B compared with control group A.

Group B rats showed a significant reduction in BEI values (50%, p<0.05) at 7 hours after MCA occlusion compared with those in group A rats, indicating a significant reduction of brain edema by TVPOB with the combined treatment of verapamil, dexamethasone, and 20% mannitol. The treatment of TVPOB with the combined three agents also produced a significant reduction of the water content in the ischemic hemisphere (p<0.05) as compared with that in the ischemic hemi-

Table 3. Effect of TVPOB With Combined Cytoprotective Agents on Brain Water Content (% H2O) After 7 Hours of Left Middle Cerebral Artery Occlusion

<table>
<thead>
<tr>
<th>Hemisphere</th>
<th>Control group (n=6)</th>
<th>TVPOB group (n=6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ischemic</td>
<td>78.73±0.11</td>
<td>77.95±0.27*</td>
</tr>
<tr>
<td>Nonischemic</td>
<td>77.50±0.16</td>
<td>77.13±0.14</td>
</tr>
</tbody>
</table>

Values are mean±SEM. TVPOB, transvenous perfusion of the brain. *p<0.05 significantly different from control group by one-way analysis of variance.
sphere in the control group by ANOVA with Tukey's test (Table 3).

Discussion
In experiment 1, we found that BBB permeability change had already occurred in the ischemic cortical areas 7 hours after MCA occlusion in the control rats (Table 1). Further worsening of the BBB permeability changes were observed by adding the vasodilating agent verapamil despite using only half of the dose (0.1 mg/kg • 2 hr⁻¹ or 0.8 µg/kg • min⁻¹) previously used in group V.³,⁶ The combined treatment comprising verapamil with either 20% mannitol or high-dose dexamethasone did not result in any beneficial effect on the BBB permeability changes in group VD rats compared with those in the control group (Figure 1). The group treated by TVPOB with the combined cytoprotective agents verapamil, dexamethasone, and 20% mannitol (group VDM) showed a significant improvement of BBB permeability changes. Because of this result, we examined further the efficacy of this combined therapeutic approach in experiment 2, in which we demonstrated that the combined treatment resulted in a significant beneficial effect for improvement of LCBF in the ischemic cortex and reduction of the size of ischemic cerebral damage even when treatment was started 5 hours after MCA occlusion in rats. Additionally, the BEI and water content of the ischemic hemisphere were significantly reduced by the combined treatment.

Most therapeutic interventions for acute focal cerebral ischemia have used various single agents that act primarily by increasing LCBF or blocking the metabolic process involved in the ischemic cascade. The beneficial effects resulted mainly through systemic administration of a cytoprotective agent given either before or immediately after occlusion.¹,²,⁴,⁶ Calcium antagonists,¹ free radical scavengers,⁴ and NMDA receptor antagonists⁵,⁷ were reported to improve LCBF or reduce the volume of ischemic cellular damage when administered before or shortly after focal ischemia in animals. Recently Morikawa et al³ reported that postischemic treatment with (S)-emopamil administered systemically within 2 hours after MCA occlusion in rats resulted in a significant reduction of cerebral infarct volume.

Results from a recent trial¹⁹ examining early treatment of acute ischemic stroke in humans indicated that when an emergency medical approach was used¹⁹ the total time from stroke onset to earliest initiation of treatment was 3–5 hours.

Experimental studies at our institution²⁰–²² indicated that a combination of cytoprotective agents may act synergistically to produce a significantly greater beneficial effect on ischemic brain than any of the agents used singly. Salgado et al²² reported that bimodal therapy with low-molecular-weight dextran and nimodipine has a beneficial effect on LCBF and ischemic volume in a rat MCA occlusion model when treatment is started within 20 minutes after occlusion.

We³,⁶ recently established a new therapeutic approach using TVPOB for acute focal ischemia in rats. Our results indicated that TVPOB with the calcium antagonist verapamil had a selective and remarkably beneficial effect on the ischemic brain when treatment was started 1 and 3 hours after MCA occlusion. This therapeutic approach with TVPOB has a much wider therapeutic time window than do previously reported approaches using the systemic administration of cytoprotective agents,¹,²,⁴,⁶ and it accommodates the practical clinical time window reported in recent clinical trial findings by Barsan et al.¹⁹ However, we observed the additional problems of ischemic brain edema associated with BBB permeability changes and production of free radicals, which generally developed 5–6 hours after the onset of ischemia.⁵,¹¹–¹² It was impossible to protect ischemic brain effectively with verapamil alone after these complications had occurred. In fact, we examined the efficacy of TVPOB with verapamil alone when treatment was started 3 hours after 4 hours of MCA occlusion in rats (i.e., 7 hours after initiation of MCA occlusion), but no significant beneficial effect was observed in that study (M. Shimauchi and Y.L. Yamamoto, unpublished observations). Furthermore, we noted worsening brain edema 7 hours after MCA occlusion with a dose of 0.1 mg/kg • 2 hr⁻¹ verapamil. We therefore reduced the dose of verapamil to half of that previously used (i.e., 0.05 mg/kg • 2 hr⁻¹) in this experiment.

To obtain effective treatment of prolonged cerebral ischemia through the use of TVPOB with a combined cytoprotective agent treatment, we also evaluated a variety of agents to counter the effects of edema and free radicals. A high dose of mannitol was known to act as a free radical scavenger, protecting against oxygen free radical damage of ischemic tissue.⁴,⁵,¹¹,¹³ It was impossible to obtain effective treatment of prolonged cerebral ischemia through the use of TVPOB with a combined cytoprotective agent treatment, we also evaluated a variety of agents to counter the effects of edema and free radicals. A high dose of mannitol was known to act as a free radical scavenger, protecting against oxygen free radical damage of ischemic tissue.⁴,⁵,¹¹,¹³

Most investigators have found that high doses of steroids alone do not improve the outcome or inhibit edema formation after experimental infarction,²⁷–³⁰ although few studies revealed that treatment with high doses have a beneficial effect on edema formation.³¹,³² Recent clinical trials have also indicated that steroids alone were not effective for ischemic stroke.³³,³⁴ However, a combined treatment of 20% mannitol (1.2–2 g/kg) and high-dose dexamethasone (4–8 mg/kg)⁸ and treatment with other cytoprotective agents⁵⁵ were found to have beneficial effects in the treatment of acute and subacute focal cerebral ischemia in rats.

In group VDM, treatment using TVPOB with verapamil, dexamethasone, and 20% mannitol resulted in extensive and significant reduction of BBB permeability change in ischemic cortex compared with treatments in the other groups (Table 1 and Figure 1). Furthermore, we confirmed that treatment using TVPOB with the combined three agents improved LCBF in the ischemic cortex (Table 2) and reduced the volume of ischemic cerebral damage (Figure 3) and brain edema in prolonged focal cerebral ischemia (Table 3). The therapeutic approach with TVPOB and the three-agent combination resulted in a significant beneficial effect in subacute focal ischemic brain when treatment was started 5 hours after MCA occlusion in rats.

Acknowledgments
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References


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**Editorial Comment**

There are two important and somewhat surprising findings in this article. The authors reported that although verapamil, mannitol, and dexamethasone individually were ineffective in protecting the brain in a focal ischemia model, when administered in combination by a retrograde transvenous route they were effective in reducing the increased permeability of the blood–brain barrier to α-aminoisobutyric acid, in reducing brain edema and brain infarct volume, and in improving cerebral perfusion. The mechanism of the action of the combined therapy is not entirely clear. For example, verapamil was given for its vasodilatory action, which was clearly evident from the improvement in cerebral blood flow. However, it may also have beneficial effects through protecting cells from calcium overload. Mannitol is of course a scavenger of hydroxyl radical, but it can have effects mediated through hemodilution and associated secondary vascular effects, or it can exert osmotic effects that lead to reduction in brain edema.
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