Temporal Thresholds of Reperfusion in the Middle Cerebral Artery Occlusion Model in Rats

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In this study, the middle cerebral artery (MCA) of adult male Sprague-Dawley rats was occluded by the modified Koizumi method to determine the temporal thresholds of reperfusion for the treatment of cerebral embolism. Regional cerebral blood flow (rCBF) and pathological findings were measured at 1 and 2 h of ischemia and after 24 h and 7 days of reperfusion following 1 or 2 h of ischemia. rCBF was decreased the most (less than 10% of control CBF) in the parietal cortex (Pcor) and the lateral caudoputamen (Lcp) at both 1 and 2 h of ischemia. There was no significant difference in rCBF in these areas between the 2 ischemic groups. The 2 h ischemia group clearly showed infarction in the area perfused by the middle cerebral artery (including the Pcor and Lcp) after 24 h and 7 days of reperfusion, while the 1 h ischemia group showed only slight infarction. These findings suggest that temporal thresholds of reperfusion in this model exist between 1 and 2 h of ischemia, and that rCBF levels during ischemia and the duration of ischemia are the most important factors in producing brain infarction.

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Patients with cerebral embolism have a poor clinical prognosis because the main trunk of the cerebral artery is occluded, which produces a large infarction in the brain. It is generally thought that thrombolytic therapy is contraindicated for embolism, and that the patient is best treated by a hyperosmotic agent after a certain period of ischemia has passed. Our experience suggests that the best therapy may be to remove the embolus and to restore cerebral blood flow early in the ischemic period. However, reperfusion often exacerbates brain edema and ischemic brain damage after several hours of ischemia. Therefore, we sought to determine the temporal threshold of reperfusion after ischemia within which ischemic brain damage can be ameliorated.

Recently, several rat models of permanent focal ischemia have been developed which result in a consistent infarction, and in which most physiological parameters are well controlled. The rat is an appropriate species for the study of focal ischemia because the ischemia is reproducible, and the animal is more easily handled than the monkey or cat. The rat model in which the middle cerebral artery (MCA) is occluded intraluminally using a silicone rubber cylinder attached to a nylon suture via the carotid artery has the particular advantage of not requiring a craniotomy, which precludes the need for an invasive surgical procedure, and reperfusion is easily induced. It is believed that this is an appropriate model of cerebral embolism in humans. In this study, regional cerebral blood flow (rCBF) and pathology were sequentially investigated using this focal

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ischemic model in rats to determine the temporal thresholds of reperfusion.

MATERIALS AND METHODS

(1) Animal Preparation

This experiment regarded to Guideline of Animal Experimentation in Nippon Medical School. Seventy eight adult male Sprague-Dawley rats weighing 250–350 g were used. They were initially anesthetized with 2% halothane. Anesthesia was then maintained by means of a face mask through which 1% halothane was administered in a 7:3 mixture of nitrous oxide and oxygen. A polyethylene catheter was inserted into the tail artery to continuously monitor arterial blood pressure and to sample blood for blood gas analysis. Blood gas and glucose levels were measured at 10 min before ischemia and at 20 min after ischemia. Arterial blood pressure was continuously monitored for 30 min, from 10 min before MCA occlusion to 20 min after MCA occlusion. During the experiment, the rectal and scalp temperatures were regulated between 37°C and 38°C using a heat lamp and a heating pad.

The rats were placed in the supine position. After a median incision of the neck skin, the left common carotid artery and the bifurcation of the internal and external carotid artery were exposed with careful conservation of the vagus nerve. The common and external carotid arteries were ligated. The left MCA was occluded by embolization, using a silicone rubber cylinder attached to 4–0 nylon surgical thread, through the left internal carotid artery. Reperfusion was achieved by releasing the surgical thread from the internal carotid artery (the modified Koizumi method1).

In this study, rats which died during reperfusion were excluded from the experiment.

(2) Regional CBF Measurement (rCBF)

rCBF was measured at various time points (at 1 h of ischemia (n=5), 1 h of ischemia with 24 h of reperfusion (n=5) or 7 days after reperfusion (n=5), and at 2 h of ischemia (n=5), 2 h of ischemia with 24 h of reperfusion (n=5) or 7 days after reperfusion (n=5)) using a 14C-iodoantipyrine quantitative autoradiographic technique (the method of Sakurada et al9). In the 1 and 2 h ischemia groups which were not reperfused, polyethylene catheters were inserted into the femoral artery and vein during the surgical procedure. After the surgical procedure, anesthesia was discontinued. In the reperfusion groups, the rats recovered after reperfusion and were allowed free access to food and water. At 2–3 h before the designated measurement time, the rats were anesthetized with 2% halothane and catheters were inserted, and anesthesia was then discontinued. One hundred fifty µCi/kg (5.55 MBq) of 14C-iodoantipyrine was infused intravenously within 30 sec. During the infusion, several arterial blood samples from the free-flowing femoral artery catheter were collected in sample tubes at 3 sec intervals. The concentration of 14C-iodoantipyrine in the blood samples was determined by a liquid scintillation counter. The rats were decapitated approximately 30 sec after the start of the infusion. The brains were quickly removed and frozen in isopentane chilled to –50°C using liquid N2. Each brain was sectioned (20 µm) in a cryostate and the sections were exposed to X-ray film with an autoradiographic carbon-14 standard (Amersham) for 2 weeks. Cerebral 14C tissue concentrations were determined by The Microcomputer Imaging Device (MCID system, Imaging Research Inc), and rCBF was measured using tissue and blood concentrations of 14C (MCID system). In a sham-operated group (n=5), a median incision was made in the neck skin, and the common and external carotid arteries were ligated, but the 4–0 nylon surgical thread was not inserted. rCBF was measured by the same autoradiographic method, and was used as a control. rCBF was expressed as a percentage of the mean control rCBF.

rCBF was measured at the parietal cortex (Pcor), frontal cortex (Fcor), lateral caudoputamen (Lcp), medial caudoputamen (Mcp), thalamus, hippocampus and substantia nigra. The anatomical regions were referenced using The Rat Brain in Stereotaxic Coordinates20.

(3) Pathological Study

Pathological observations were made at 1 h of ischemia (n=6), 1 h of ischemia with 24 h of reperfusion (n=6) or 7 days after
TABLE 1 PHYSIOLOGICAL PARAMETERS

<table>
<thead>
<tr>
<th></th>
<th>1 h ischemia group</th>
<th>2 h ischemia group</th>
<th>Control (n=11)</th>
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<tbody>
<tr>
<td></td>
<td>Before ischemia (n=33)</td>
<td>After ischemia (n=33)</td>
<td>Before ischemia (n=33)</td>
</tr>
<tr>
<td>Rectal temperature (°C)</td>
<td>37.5±0.6</td>
<td>37.2±0.5</td>
<td>37.7±0.4</td>
</tr>
<tr>
<td>Scalp temperature (°C)</td>
<td>37.6±0.3</td>
<td>37.4±0.2</td>
<td>37.5±0.3</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
<td>104.4±8.2</td>
<td>104.6±6.7</td>
<td>98.7±10.9</td>
</tr>
<tr>
<td>Pco₂ (mmHg)</td>
<td>37.1±5.6</td>
<td>40.1±8.8</td>
<td>35.6±10.8</td>
</tr>
<tr>
<td>Po₂ (mmHg)</td>
<td>115.7±17.7</td>
<td>116.3±20.9</td>
<td>100.8±10.9</td>
</tr>
<tr>
<td>pH</td>
<td>7.39±0.05</td>
<td>7.38±0.07</td>
<td>7.41±0.04</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>103.7±14.8</td>
<td>103.1±10.1</td>
<td>115.4±7.6</td>
</tr>
</tbody>
</table>

Values are mean±SD
There were no difference among each ischemia group and control.

![Fig. 1. Regional cerebral blood flow autoradiograms in coronal sections at the level of the caudoputamen after 1 h (left) or 2 h (right) of ischemia are shown, rCBF decreased the most in the core areas (arrows). In the core areas, there was no difference in rCBF between 1 and 2 h of ischemia. However, in the watershed areas (arrowheads), rCBF after 1 h of ischemia was higher than that after 2 h.](image)

reperfusion (n=6), and at 2 h of ischemia (n=6), 2 h of ischemia with 24 h of reperfusion (n=6) or 7 days after reperfusion (n=6). The sham-operated group (n=6) was used as a control. At the designated times, perfusion-fixation was performed with 4% formaldehyde via the ascending aorta and the brains were removed from the skulls. The brain sections (6 µm) were stained with hematoxylin and eosin. To measure the size of the infarction at 7 days after reperfusion, outlines of the infarct were drawn on brain maps at 4 levels (Bregma +1.0 mm, −1.0 mm, −3.0 mm, and −5.0 mm, where the bregma is the intersection between the sagittal suture and the coronal suture. Bregma +1.0 mm=1.0 mm anterior to the Bregma, and Bregma −1.0 mm=1.0 mm posterior to the Bregma.) while observing the specimen by light microscopy. The size of the infarction was measured at these levels using an image-analyzer (The MCID system). The size of the infarction in each section was expressed as a percentage of infarcted area of the total coronal section.

(4) Statistical Analysis
The data were expressed as the mean±SD. Statistical analysis was performed using Sheffe’s Multiple Comparison test to determine significant differences between the groups. A level of p<0.05 was considered significant.

RESULTS

(1) Physiological Parameters
The physiological data are presented in

Temporal Thresholds of Reperfusion

| TABLE II | REGIONAL CEREBRAL BLOOD FLOW (%) | (%)
<table>
<thead>
<tr>
<th></th>
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</thead>
<tbody>
<tr>
<td>MCAO</td>
<td>MCAO: middle cerebral artery occlusion.</td>
<td></td>
</tr>
<tr>
<td>1 h (n=5)</td>
<td>1h ischemia (n=5)</td>
<td>2 h (n=5)</td>
</tr>
<tr>
<td>Frontal Cortex</td>
<td>45.2±5.6</td>
<td>7.0±2.7</td>
</tr>
<tr>
<td>Parietal Cortex</td>
<td>68.7±8.0</td>
<td>153.4±66.6</td>
</tr>
<tr>
<td>Lateral Caudoputamen</td>
<td>73.4±19.2</td>
<td>131.4±66.6</td>
</tr>
<tr>
<td>Medial Caudoputamen</td>
<td>80.9±23.2</td>
<td>94.5±23.2</td>
</tr>
<tr>
<td>Thalamus</td>
<td>84.3±23.2</td>
<td>94.5±23.2</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>36.1±16.8</td>
<td>88.7±13.9</td>
</tr>
<tr>
<td>Substantia Nigra</td>
<td>84.3±23.2</td>
<td>94.5±23.2</td>
</tr>
</tbody>
</table>

Fig. 2. rCBF levels at 1 h (n=5) and 2 h (n=5) of ischemia in the Parietal cortex (Pcor), Frontal cortex (Fcor), Lateral caudoputamen (Lcp), and Medial caudoputamen (Mcp). rCBF decreased prominently in the Pcor and Lcp in both the 1 and 2 h ischemia groups, and there was no difference in rCBF between the two groups. However, in the Fcor and HCP rCBF after 1 h of ischemic was higher than that after 2 h. Bar graphs are mean±SD of cerebral blood flow levels. N.S.: not significant.

Table I. Blood glucose, pH, PO2, pCO2, mean arterial blood pressure, scalp and rectal temperatures were measured. There were no differences in any of these parameters among the five groups.

In this experiment, none of the animals in the 1 h ischemia group died, while one of the 7 rats in the 2 h ischemia group with 7 days of reperfusion (pathological study) died 2 days after reperfusion.

(2) Regional Cerebral Blood Flow (rCBF)

The mean control CBF was measured in the designated areas. Mean control CBF in the Pcor and Lcp was 175.6, and 155.0 ml/100 g per min; that in the Fcor and Mcp was 155.0, and 167.6 ml/100 g per min; and that in the thalamus, hippocampus, and substantia nigra was 154.8, 86.8, and 119.2 ml/100 g per min, respectively.

Autoradiograms performed at 1 and 2 h of ischemia are shown in Fig. 1, and rCBF values are shown in Table II. The CBF values in the Pcor and Lcp regions were 7.0±2.7 and 9.1±2.2% at 1 h of ischemia and 4.3±1.5 and 6.2±0.9% at 2 h of ischemia, respectively. rCBF decreased the most in these regions, compared to the control CBF and the other regions. There was no
difference in rCBF between the two ischemic groups in these two regions (Fig. 2). rCBF in the Pcor and Mcp, which were watershed areas, decreased to 45.2±5.6 and 43.7±6.7% at 1 h of ischemia and to 21.4±3.0 and 15.8±2.5% at 2 h of ischemia, compared to the control, and there was a significant difference in rCBF between the two ischemic groups in these two regions (Fig. 2).

At 24 h of reperfusion, rCBF was 127.0±15.7 and 147±63.9% at 1 h of ischemia and 131.4±66.6 and 156.3±52.3% at 2 h of ischemia in the Pcor and Lcp, respectively, which reflected hyperperfusion in both groups. There was no difference between the two groups in these two regions (Fig. 3). In the Fcor and Mcp, rCBF was 66.7±20.5 and 95.0±45.0% at 1 h of ischemia, and 73.4±19.2 and 117.3±61.3% at 2 h of ischemia, with no difference between the two groups in these two regions (Fig. 3).

At 7 days after reperfusion, rCBF in the Pcor and Lcp was 65.8±15.7 and 84.3±23.2% at 1 h of ischemia, and 36.1±16.8 and 88.7±13.9% at 2 h of ischemia, respectively. There was no difference between the two groups in these two regions (Fig. 4). In the Fcor and Mcp, rCBF was 80.7±8.0 and 81.4±29.0% at 1 h of ischemia, and 42.8±15.5 and 43.6±11.7% at 2 h of ischemia, respectively, with a significant difference between the two groups (Fig. 4).

In the 1 h ischemia group, there was no difference in rCBF between Pcor and Lcp, or between Mcp and Fcor at any of the time points. In the 2 h ischemia group, there was no difference in rCBF between Mcp and Fcor at any of the time points, while there was a significant difference in rCBF between Pcor and Lcp 7 days after reperfusion.

In the thalamus, rCBF decreased to 69.6±17.1% of the control at 1 h of ischemia and to 58.4±20.7% at 2 h of ischemia. After reperfusion, rCBF did not return to normal control levels in the 2 h ischemia group (Table II). In the hippocampus, rCBF was 80.7±29.1% at 1 h of ischemia and 47.0±6.5% at 2 h of ischemia. After reperfusion, rCBF was maintained at about 80% of that in the control (Table II). In the substantia nigra, rCBF was 139.7±30.9% at 1 h of ischemia and 108.1±9.6 at 2 h of ischemia, and rCBF tended to increase. The levels of rCBF in both groups varied from normoperfusion to hyperperfusion at 24 h after reperfusion (Table II).

(3) Pathological Observation
Infarcted tissue was not clearly observed at 1 or 2 h of ischemia. However, ischemic cell changes, which appeared as shrunken and triangular neurons with dark-stained...
nuclei, were moderately observed in the Lcp and Pcor at 2 h of ischemia. In contrast, only slight ischemic cell changes were observed in the Lcp and the Pcor at 1 h of ischemia. After 24 h of reperfusion, typical ischemic changes with most of the neurons shrunken and triangular, as well as spongy changes, were observed in the 2 h ischemia group in the Pcor and Lcp, and the ischemic lesion was clearly demarcated. In the 1 h group, ischemic cell changes were observed in the Lcp, but only slightly in the Pcor. Ischemic cell damage was observed over significantly less area in the 1 h ischemia group than in the 2 h ischemia group. After 7 days of reperfusion in the 2 h group, infarction was observed in the areas perfused by the middle cerebral artery (including the Lcp and Pcor) in all of the rats. Infiltration of macrophages within and at the border of the infarct was observed and macrophages which had phagocytized necrotic tissue were also observed (Fig. 5). In the 1 h group, neuronal ischemic changes were observed in the Lcp, and only slightly in the Pcor (Fig. 5).

The size of the infarction in the 1 and 2 h ischemia groups after 7 days of reperfusion is shown in Table III. At each level, the size of the infarction in the 1 h group was significantly less than that in the 2 h group.

In the Mcp and Pcor, which are watershed areas between the anterior cerebral artery (ACA) and the middle cerebral artery, the border between normal and ischemic tissue was clearly observed after 24 h and 7 days of reperfusion following 2 h of ischemia. In contrast, the border was not clear in the 1 h group, and ischemic tissue was not observed in these areas. In the thalamus, hippocampus and substantia nigra, which are not
perfused by the middle cerebral artery, infarction was not clearly observed.

**DISCUSSION**

This study was performed to determine temporal thresholds of reperfusion in a novel middle cerebral artery occlusion model in rats using a modification of Koizumi's method, which is convenient for inducing focal cerebral ischemia.

MCA occlusion caused the greatest reduction in CBF in the Pcor and Lcp at 1 and 2 h of ischemia, which shows that the Pcor and Lcp are ischemic core areas in this model. In these areas, CBF levels were less than 10% of the control CBF, approximately 5–15 ml/100 g per min, in both the 1 and 2 h ischemia groups (Table II). In a preliminary study, we sequentially measured rCBF in the Lcp at 30, 60, and 120 min after MCA occlusion using the hydrogen clearance method. rCBF fell to 9.7 ± 0.9 at 30 min, 9.6 ± 1.0 at 60 min and 9.8 ± 0.9 ml/100 g/min at 120 min after ischemia. These results showed that the reduction in rCBF occurred in the early phase of ischemia and continued throughout ischemia.

In the present study, when the duration of ischemia was extended to 2 h, infarction occurred in the ischemic core areas. Previous studies have attempted to determine the flow and time thresholds for ischemic brain damage. Tamura et al reported that in the cat MCA occlusion model, CBF levels below 12–15 ml/100 g per min for 2 h were associated with ischemic brain damage, even though reperfusion was performed. Morawetz et al reported that the flow threshold for infarction was 12 ml/100 g per min in the monkey MCA occlusion model. Jones et al found that tissue with blood flow below 10–12 ml/100 g per min was infarcted after 2–3 h while that with flow below 17–18 ml/100 g per min was infarcted if occlusion persisted for 2 weeks. Using a method similar to that used here, Memezawa et al found that 60 min of transient ischemia induced cortical and lateral caudoputamen infarction in every rat tested, and there was no significant difference in the size of the infarction between the 120 min ischemia group and the 180 min group. In this experiment, the Lcp and Pcor showed ischemic cell changes or infarction in both the 1 and 2 h ischemia groups. In particular, the 2 h group showed infarction in the area perfused by the middle cerebral artery, while the ischemic change in the Pcor in the 1 h group was only slight. Therefore, the size of the infarction was significantly different between the 1 and 2 h ischemia groups. Our data are similar to those of Memezawa, and suggest that the temporal thresholds in the core areas for infarction in this model are between 1 and 2 h.

In the 1 h ischemia group, ischemic cell changes in the Lcp were observed at 24 h and 7 days after reperfusion in all rats. In contrast, there were only slight ischemic cell changes in the Pcor. With reference to these regional differences after 1 h of transient ischemia, Abe et al showed using a rat focal ischemic model that a profound inhibition of protein synthesis and the formation of brain edema began sooner in the lateral caudate than in the cortex, and continued long after reperfusion, although CBF during occlusion and in the early period of reperfusion were similar. They noted that this regional difference might be due to a distinctive response to ischemic stress. In our measurement of rCBF, the 1 h ischemia
group showed no differences in CBF between the Pcor and Lcp at any of the time points. In the 2 h ischemia group, no pathological differences were observed between the Pcor and Lcp. These results show that transient ischemia for 2 h in this model is sufficient for infarction in the occluded area.

After reperfusion, rCBF in the Pcor and Lcp was restored, but to varying degrees ranging from normal to hyperperfusion. However, there was no difference in the rCBF between the 1 and 2 h ischemia groups. Considering rCBF levels during and after ischemia, our results suggest that rCBF levels during ischemia and the duration of ischemia are the most important factors in brain infarction.

In the Mcp and Feor, which are watershed areas between the ACA and the MCA, rCBF levels were different at 1 and 2 h of ischemia. In the 2 h group, rCBF levels were about 40% of the control after 7 days of reperfusion. The hypoperfusion observed in this experiment may be due to the location of these regions on the border between ischemic and normal tissue. In contrast, in the 1 h group, rCBF levels in these areas were maintained at about 80% of the control after 7 days of reperfusion and ischemic changes were not observed. Therefore, the difference in rCBF was attributed to the pathological findings, which showed that the tissue in these areas might be salvaged if MCA occlusion is removed prior to 2 h.

Infarction was not clearly observed in the thalamus, which is not perfused by the middle cerebral artery. However, rCBF in this area was reduced at both 1 and 2 h of ischemia, compared to the control. In particular, after 7 days of reperfusion following 2 h of ischemia, rCBF was significantly less than that after 7 days of reperfusion following 1 h of ischemia. It has been previously reported that focal brain ischemia induced a change in blood flow in areas remote from the ischemic lesion: i.e., diaischisis. In this study, after 2 h of transient ischemia, infarction occurred in the caudoputamen and cortex which was perfused by the middle cerebral artery. The decrease in rCBF in the thalamus is thought to be due to a secondary interruption of the neuronal connection, since a rich nerve fiber connection exists between the thalamus and the cortex. In the hippocampus and substantia nigra, the change of in CBF may possibly be due to a remote effect of focal ischemia. However, it is unclear from this study whether 1 and 2 h of transient ischemia yield a different prognosis.

In summary, this study clarifies that the Pcor and Lcp are core areas in this ischemic model. In these core areas, there was no difference in rCBF between the 1 and 2 h ischemia groups at each time point. However, there were significant differences in pathological findings and infarction size between the two groups. These findings suggest that temporal thresholds of reperfusion occur between 1 and 2 h of ischemia, and that flow thresholds for inducing infarction are about 10% of the control values.

Acknowledgments

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