Neutral red staining was evaluated as an acute outcome assessment method in rat models of cerebral ischemia by comparison with histological infarction volume. Fischer 344 rats (n = 48) were used in three different models of middle cerebral artery (MCA) occlusion: proximal MCA occlusion (n = 16), distal MCA occlusion followed by ipsilateral common carotid artery (CCA) occlusion (distal MCA/CCA occlusion, n = 15), and MCA occlusion with an intravascularly introduced 4-0 nylon suture (intravascular MCA occlusion, n = 17). At 1 hour, 2 hours, and 4 hours after MCA occlusion, animals were injected with 2.5 ml of 4% neutral red solution via the femoral vein, and then sacrificed. Proximal MCA occlusion caused a neutral red defect volume in the cortex which correlated well with histological infarction volume at 4 hours (r = 0.88, p < 0.05), and in the caudate which correlated well with infarction volume at 4 hours (r = 0.94, p < 0.01). Distal MCA/CCA occlusion caused a neutral red defect volume in the cortex larger than the histological infarction volume (4 hrs: 88.6 ± 11.8 vs. 74.3 ± 17.4 mm³, p < 0.05) but closely correlated with the infarction volume at 4 hours (r = 0.81, p < 0.05). Intravascular MCA occlusion caused a neutral red defect volume in only two of 17 animals after 1-4 hours, which correlated well with the absence of histological evidence of infarction. Neutral red staining is a simple method for assessing the acute outcome of focal cerebral ischemia as early as 4 hours after the onset, in an appropriate model of cerebral ischemia.

Key words: cerebral ischemia, animal models, neutral red, pathology

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Introduction

The histological detection of cerebral infarction may require up to 4-6 hours after experimental focal cerebral ischemia is induced, depending on the fixation technique. Critical physiological parameters such as temperature, blood gases, or blood glucose may vary within this period and have a significant effect on infarction size. An experimental model of focal cerebral ischemia allowing control of these physiological parameters and use of an acute outcome assessment method should reduce inter-animal variation. A rapid acting, simple cellular marker for quantifying ischemic injury would be most useful as an acute outcome assessment method.

Neutral red is a nontoxic vital dye that can pass across the blood brain barrier and has been used as an intracellular pH indicator. Neutral red staining diminishes in intensity according to cerebral blood flow (CBF) reduction in an infarcted area. The neutral red staining pattern was closely correlated with the area of decreased CBF measured by 14C-ioodoantipyrine autoradiography 2 hours after middle cerebral artery (MCA) occlusion, suggesting that neutral red can detect reduction in CBF and predict tissue at risk of infarction in the early stages after focal cerebral ischemia.

Our study investigated neutral red staining after MCA occlusion using three different rat models of focal cerebral ischemia for comparison with the region of infarction, to evaluate neutral red staining as a method for assessing the acute outcome of focal cerebral ischemia.

Materials and Methods

I. Surgical preparations

Forty-eight male Fischer 344 rats (202-330 g) were
allowed free access to food and water before the experiment. The rats were anesthetized with 2% halothane, followed by tracheostomy and mechanical ventilation (Harvard rodent ventilator model 683; Harvard Apparatus Co., South Natick, Mass., U.S.A.) with a 0.5–1.0% halothane, nitrous oxide/oxygen (70%:30%) mixture to ensure normoxia and normocapnia. Polyethylene catheters (PE-50) were introduced into a femoral artery and a femoral vein to allow continuous monitoring of blood pressure, measurement of arterial blood gases, and administration of fluids. The rectal temperature was monitored and the animals were kept normothermic (37.0 ± 1.0°C) by heating with an overhead lamp.

II. Models of focal cerebral ischemia

i) Proximal MCA occlusion was performed in 16 rats according to Tamura et al.@ with minor modifications. Under the operating microscope, the left MCA was exposed transcranially without removal of the zygomatic bone, and occluded 2 mm proximal to the olfactory tract with bipolar coagulation.

ii) Distal MCA occlusion with common carotid artery (CCA) occlusion (distal MCA/CCA occlusion) was performed in 15 rats as described previously.2,3) Under the operating microscope, the left distal MCA was exposed just distal to the rhinal fissure through a 2 mm burr hole, and was clipped (#1 microclip; Codman, Boston, Mass., U.S.A.) followed by ipsilateral CCA occlusion using a microclip.

iii) MCA with an intravascularly introduced 4–0 nylon suture without craniectomy (intravascular MCA occlusion) in 17 rats according to Longa et al.11 The right CCA was exposed through the tracheostomy incision. The right internal carotid artery (ICA) and the CCA were temporarily occluded. A 4-0 nylon suture was introduced into the ECA lumen through an incision. A 6-0 silk suture was tied around the nylon suture in the ECA to prevent bleeding. The temporary clips on the CCA and ICA were removed and the 4-0 nylon suture was advanced from the ECA along the ICA to the anterior cerebral artery to block the origin of the MCA. The length of the introduced suture was 17.5 ± 1.2 mm (mean ± SD).

III. Neutral red injection and brain fixation

Four percent neutral red (toluylene red: 3-amino-7-dimethyl-amino-2-methylphenazine hydrochloride, 54% purity, Cl 50040; Sigma Chemical Co., St. Louis, Mo., U.S.A.) was prepared in lactated Ringer’s solution. To prevent hypotension, the neutral red solution was filtered and the pH adjusted according to Selman et al.17) The neutral red solution (2.5 ml) was injected into the rats over 10 minutes with an infusion pump (Model 903; Harvard Apparatus Co.) via the femoral vein at 1 hour, 2 hours, or 4 hours after MCA occlusion. All rats were rapidly decapitated, and the brains were removed and stored in 10% formalin for over 24 hours.

The rat forebrains were cut into eight pre-selected coronal sections according to the atlas of Konig and Klippel.9) The area unstained by neutral red (Fig. 1) was recorded by hand on a template.4) The brain slices were embedded in paraffin wax, cut into sections (7–8 µm), and stained with hematoxylin and eosin (HE) for histological examination. Neutral red does not affect the HE staining.12) Cerebral infarction was defined histologically as reduced HE background staining or significant pyknotic neuronal changes or loss. No histological examination was performed on specimens from the animals sacrificed 1 hour after the onset of ischemia. The infarcted area was recorded by hand on templates. The areas unstained by neutral red demonstrating infarction were measured on the templates using image analysis software (MCID M1 4.12; Imaging Research Inc., St. Catherines, Ontario, Canada), and the brain volumes affected were calculated (neutral red defect and infarction volumes, respectively).

IV. Statistical analysis

Physiological parameters were averaged over the experimental time period, and compared by one way analysis of variance (ANOVA). The correlation coefficients between neutral red defect and infarction

Fig. 1 Coronal section of rat brain 4 hours after proximal MCA occlusion. The neutral red staining defect is the white area in the cortex and the caudate.
volumes were calculated, averaged within an experimental group, and analyzed by ANOVA. The unpaired t-test was used to evaluate differences. p < 0.05 was considered significant. All values are expressed as mean ± SD.

### Results

There were no significant differences between the groups in temperature, PCO$_2$, pH, and mean blood pressure (Table 1). The mean neutral red defect volumes and infarction volumes after MCA occlusion are summarized in Table 2.

Proximal MCA occlusion caused a neutral red defect volume in the cortex which was maximum after 1 hour, and became smaller after 2 and 4 hours. The neutral red defect volume in the caudate remained the same after 1, 2, and 4 hours. The infarction volume gradually increased after 2 and 4 hours. The neutral red defect volume correlated well with the infarction volume 4 hours after ischemia (cortex: $y = 12.6 + 0.85x$, $r = 0.88$, $p < 0.05$; caudate: $y = 0.84 + 1.04x$, $r = 0.94$, $p < 0.01$) (Fig. 2). Moreover, 4 hours after onset of proximal MCA occlusion, the neutral red defect volumes in the cortex and caudate were not significantly different from the infarction volumes (Table 2).

Distal MCA/CCA occlusion caused a neutral red defect volume in the cortex which remained similar after 1, 2, and 4 hours. The infarction volume showed a tendency to increase after 2 and 4 hours, but this was not significant. The neutral red defect volume was closely correlated with the infarction volume 4 hours after distal MCA/CCA occlusion ($y = -31.9 + 1.20x$, $r = 0.81$, $p < 0.05$) (Fig. 3), but the neutral red defect volume was significantly greater than the infarction volume after 4 hours ($p < 0.05$) (Table 2).

Intravascular MCA occlusion did not cause infarction at any time after ischemia, which correlated well with neutral red staining, in which minor defects were detected in only two of 17 animals.

### Table 1  Physiological parameters

<table>
<thead>
<tr>
<th>Time after ischemia (hrs)</th>
<th>No. of rats</th>
<th>Temperature (°C)</th>
<th>PCO$_2$ (mmHg)</th>
<th>PO$_2$ (mmHg)</th>
<th>pH</th>
<th>Mean arterial pressure (mmHg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proximal MCA occlusion:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>6</td>
<td>37.3 ± 0.3</td>
<td>35.7 ± 4.2</td>
<td>171 ± 45</td>
<td>7.37 ± 0.03</td>
<td>83 ± 12</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>37.5 ± 0.5</td>
<td>36.9 ± 1.8</td>
<td>146 ± 22</td>
<td>7.40 ± 0.04</td>
<td>82 ± 6</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>37.6 ± 0.5</td>
<td>35.3 ± 2.1</td>
<td>146 ± 13</td>
<td>7.41 ± 0.05</td>
<td>87 ± 12</td>
</tr>
<tr>
<td>Distal MCA/CCA occlusion:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>36.9 ± 0.2</td>
<td>36.4 ± 1.3</td>
<td>143 ± 26</td>
<td>7.40 ± 0.07</td>
<td>93 ± 9</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>37.2 ± 0.6</td>
<td>36.4 ± 4.7</td>
<td>154 ± 33</td>
<td>7.44 ± 0.03</td>
<td>92 ± 14</td>
</tr>
<tr>
<td>4</td>
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<td>37.2 ± 0.7</td>
<td>34.4 ± 4.6</td>
<td>125 ± 6*</td>
<td>7.41 ± 0.04</td>
<td>95 ± 13</td>
</tr>
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<td>Intravascular MCA occlusion:</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>37.2 ± 0.5</td>
<td>34.6 ± 3.4</td>
<td>187 ± 45</td>
<td>7.40 ± 0.07</td>
<td>87 ± 15</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>37.1 ± 0.6</td>
<td>36.9 ± 4.8</td>
<td>131 ± 14</td>
<td>7.40 ± 0.04</td>
<td>91 ± 11</td>
</tr>
<tr>
<td>4</td>
<td>8</td>
<td>37.5 ± 0.4</td>
<td>36.4 ± 1.8</td>
<td>142 ± 18</td>
<td>7.38 ± 0.07</td>
<td>89 ± 8</td>
</tr>
</tbody>
</table>

Values are mean ± SD. *p < 0.05 vs. 1 and 2 hours PO$_2$ in distal MCA/CCA occlusion.

### Table 2  Neutral red (NR) defect and infarction volumes

<table>
<thead>
<tr>
<th>Region</th>
<th>1 hour</th>
<th>2 hours</th>
<th>4 hours</th>
<th></th>
<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>NR defect</td>
<td>Infarction</td>
<td>NR defect</td>
<td>Infarction</td>
<td>NR defect</td>
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<td></td>
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<td></td>
</tr>
<tr>
<td>Proximal MCA occlusion:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cortex</td>
<td>92.9 ± 9.5*</td>
<td>ND</td>
<td>59.6 ± 5.0</td>
<td>54.0 ± 13.0</td>
<td>58.6 ± 29.1</td>
</tr>
<tr>
<td>caudate</td>
<td>9.5 ± 2.9</td>
<td>ND</td>
<td>11.1 ± 3.7</td>
<td>9.8 ± 3.6</td>
<td>9.6 ± 5.2</td>
</tr>
<tr>
<td>Distal MCA/CCA occlusion:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cortex</td>
<td>82.3 ± 13.3</td>
<td>ND</td>
<td>73.1 ± 20.2</td>
<td>60.3 ± 11.3</td>
<td>88.6 ± 11.8**</td>
</tr>
</tbody>
</table>

Values are mean ± SD (unit mm$^3$). *p < 0.05 vs. NR defect at 2 and 4 hours. **p < 0.05 vs. infarction at 4 hours. ND: not done.

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Fig. 2 Correlation between neutral red (NR) defect and infarction volumes in the cortex (left: $y = 12.6 + 0.85x$, $r = 0.88$, $p < 0.05$) and caudate (right: $y = 0.84 + 1.04x$, $r = 0.94$, $p < 0.01$) 4 hours after proximal MCA occlusion.

Fig. 3 Correlation between neutral red (NR) defect and infarction volumes in the cortex 4 hours after distal MCA/CCA occlusion ($y = -31.9 + 1.20x$, $r = 0.81$, $p < 0.05$).

**Discussion**

The various rat models of MCA occlusion are known to cause infarction reliably, but the different MCA occlusion models result in different infarction patterns judging from the acute outcome. We therefore used three different MCA occlusion models to evaluate neutral red staining as an acute outcome assessment method. Proximal MCA occlusion causes infarction in the caudate and the cortex. Tyson et al. reported that a decrease in CBF to <25 ml/100 g/min measured by $^{14}$C-iodoantipyrine autoradiography correlated well with the infarction area 4 hours after proximal MCA occlusion. Therefore, this model can be used to investigate infarction in two different areas (the caudate and the cortex) in the acute stage. Distal MCA/CCA occlusion causes infarction only in the cortex, but this technique is very simple and reproducible. Intravascular MCA occlusion causes infarction of the basal ganglia and the cortex without damage to the brain, but the location of the tip of the suture cannot be monitored, so this method requires highly developed technique.

Our study shows that neutral red staining can predict histological damage as early as 2 hours after focal cerebral ischemia. Neutral red staining at earlier than 2 hours overestimated the infarction volume, possibly due to the dependence of neutral red staining on blood flow as well as cell viability. Nonquantitative neutral red injection may permit recognition of defect areas where CBF is greater than 25 ml/100 g/min and where neuronal death will not occur. We found that the neutral red defect volume was consistently greater than the infarction volume at 4 hours after distal MCA/CCA occlusion. A possible explanation is the use of standard fixation methodology, which may have underestimated infarction compared to perfusion fixation techniques.
Neutral red staining is affected by the model of focal ischemia. Models where the evolution of infarction is more prolonged, such as MCA occlusion with an intravascularly introduced nylon suture, may not permit accurate identification of infarcted tissue by neutral red injection.

Other outcome assessment methods can detect early cellular changes after focal cerebral ischemia. Magnetic resonance imaging and magnetic resonance spectroscopy can detect infarction areas and metabolic changes in the ischemic area in the acute stage, and can also demonstrate dynamic changes in an individual. The 3H-forskoline binding method can detect ischemic areas in the focal cerebral ischemia model as early as 30 minutes after the onset of ischemia, possibly by detecting changes in the second messenger system in the acute ischemic area. However, these acute outcome assessment methods are expensive and require dedicated, skilled technicians.

Neutral red injection is inexpensive, immediately visible, and has no risk of radioactive contamination. A simple venous neutral red injection, which detects early ischemic changes within 2–4 hours, can be used to study the neuroprotective actions of agents in ischemia, reperfusion injuries, or the evolution of related pathology. This technique is reliable and more time and cost efficient provided an appropriate focal cerebral ischemia model is used.

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References


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