Prediction of Cerebral Ischemia by Ophthalmoscopy After Carotid Occlusion in Gerbils

Jo A. Oostveen, MS; Ken Timby, CRA; and Lawrence R. Williams, PhD

Background and Purpose: The Mongolian gerbil provides a unique model of unilateral focal cerebral ischemia because of the lack of posterior communicating arteries in all gerbils as well as an absence of an anterior communicating artery in approximately 20% of the gerbil population. It is unclear how to identify unequivocally the subpopulation of animals that would suffer a severe focal cerebral ischemia after unilateral carotid occlusion.

Methods: Ninety-three male gerbils were exposed to unilateral occlusion of the right common carotid artery. The severity of neuronal loss was evaluated histologically in gerbils selected as having significant focal ischemia based on either behavioral criteria (i.e., the demonstration of stereotypical behavior within 1 hour after occlusion) or ophthalmoscopic criteria (i.e., interruption of the retinal arterial perfusion within 10 minutes of carotid ligation as assessed with an ophthalmoscope). After 3 hours of unilateral carotid occlusion, cerebral blood flow was reinstated for 24 hours before fixation for histological analysis. The viability of the CA1 region of the hippocampus, lateral cortex, and medial cortex was scored on a scale of 0-4 based on the percentage of apparent neuronal loss (e.g., 0, no damage; 4, >75% damage (the Viability Index)).

Results: Twenty-eight percent of the gerbils met the behavioral selection criteria, and 17% met the ophthalmoscopic criteria. In the specimens selected by behavioral criteria (n=7), 30% demonstrated no evidence of postischemic neuronal loss; the mean±SEM Viability Index scores for CA1, lateral cortex, and medial cortex were 1.6±0.6, 1.0±0.3, and 0.3±0.2, respectively. Of the animals selected by ophthalmoscopic criteria (n = 12), 100% had severe ischemic tissue damage to the ipsilateral hemisphere; the Viability Index scores for CA1, lateral cortex, and medial cortex were 3.5±0.1, 3.1 ±0.2, and 1.2±0.2, respectively; all scores were significantly larger than those observed in the behaviorally selected group.

Conclusions: Selection of animals by ophthalmoscopic criteria provides a reliable, consistent method to predict animals with severe focal cerebral ischemia. (Stroke 1992;23:1588-1594)

KEY WORDS • carotid arteries • cerebral ischemia • retina • gerbils

The Mongolian gerbil, Meriones unguiculatus, is widely used to model human global and focal cerebral ischemia because the gerbil lacks a circle of Willis.1-3 The posterior communicating arteries, which in all other mammals would normally connect the two vertebral arteries posteriorly (through the basilar) and the two internal carotid arteries anteriorly, are completely absent in essentially all gerbils. Thus, bilateral carotid occlusion results in global forebrain ischemia.4-6 In addition, the gerbil phenotype expresses varying degrees of anterior anastomotic collateral vascular connections.3-4 After a unilateral carotid occlusion (UCO), the degree of hemispheric ischemia correlates with the degree of anastomoses between the anterior cerebral arteries.7-9 In 20–30% of the gerbil population, an anterior anastomosis is completely absent, and UCO results in severe focal cerebral ischemia.10,11

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The gerbil UCO model of focal cerebral ischemia offers several advantages, including the ability to study transient focal ischemia and the inclusion of an internal control in the contralateral hemisphere. However, it can be difficult to predict the insufficiency of anterior anastomotic and collateral circulation and thus the degree of hemispheric ischemia during the UCO. A method has not been defined that would unequivocally identify, antemortem, those gerbils lacking the anterior vascular anastomoses to sustain the anterior frontal hemisphere. Braughler and Lainer10 used specific behavioral aberrations to identify animals with UCO-induced cerebral dysfunction. This method of selection was also used by Hall et al.11 Other criteria that have been used include observations of the diameter and appearance of the carotid artery after temporary occlusion12 and measurement of threshold arterial back pressure.13 A possibly more convenient method for routine animal preparation would be the observation of changes of vascular perfusion in the retina ipsilateral to the UCO.
After postmortem examination, Levine et al. found ischemic damage to the retina and cerebrum after permanent UCO. Variable degrees of ischemic damage were observed, but the degree of retinal destruction correlated directly with the extent of brain necrosis. The central artery of the retina branches from the ophthalmic artery, which is the first branch of the internal carotid artery. Theoretically, a loss of retinal vascular perfusion after UCO would indicate lack of anterior anastomotic collateral circulation from the contralateral carotid and would predict a relatively complete focal ischemia to the ipsilateral hemisphere and a maximum cerebral ischemic insult. Delbarre et al. reported that gerbils identified as having an absence of retinal blood flow after UCO developed severe neurological signs.

Preliminary experiments in our laboratory selected gerbils based on behavioral criteria. Histological analysis of these animals indicated that the severity of damage to the ipsilateral hemisphere was inconsistent; i.e., the degree of tissue damage was more variable than anticipated. For the present study, the variability of ischemic tissue damage was compared in gerbils selected according to behavioral and ophthalmoscopic criteria, respectively.

Materials and Methods

Ninety-three male Mongolian gerbils (Tumblebrook Farms, West Brookfield, Mass.) weighing 45–60 g were used for these experiments. Animals were housed in small groups in a room controlled for temperature (22°C) with a daily photoperiod of 12 hours of light between 6 AM and 6 PM. Each animal had free access to water and was fed ad libitum on a standard laboratory diet (Purina Rodent Chow 5001, Richmond, Ind.). Twenty-three and 70 animals were used for selection by behavioral and ophthalmoscopic criteria, respectively. For all surgical procedures, animals were anesthetized with methoxyflurane.

A ventral midline cervical incision was made, and the right common carotid artery was exposed and isolated. The artery was clamped with a microaneurysm clip (Roboz Surgical) or a microvascular clamp (Fine Science Tools), and the incision was closed with wound clips. The animals were placed in a box warmed to 37°C until recovery (approximately 10 minutes). After 3 hours of occlusion, the gerbils were briefly reanesthetized, and the clamp was removed from the right common carotid artery. The artery was visually examined to confirm reperfusion. The incision was closed with Super glue, and the animals were placed in individual cages.

After UCO, the neurological status of the animals was observed and scored at 1 hour after occlusion. Animals were assessed according to the following scale modified from Braughler and Lainer: 15

- Severe: circling, torso curvature, running fits, and barrel rolls; moderate: mild circling and torso curvature; mild: inability to walk and go around the cage.

Only animals designated as severe met the selection criteria and were assumed to lack anterior anastomotic vasculature and thus to have significant unilateral cerebral ischemia. Only these animals were used for histological evaluation.

Immediately after occlusion of the right common carotid artery, one drop of 1% tropicamide (Henry Schein, Inc., Port Washington, N.Y.) was placed in each eye to dilate the eye and facilitate observation of the retina. At 10 minutes after occlusion, retinal perfusion was assessed with a Welch-Allyn Model 111 ophthalmoscope (Henry Schein) using settings found optimal for the gerbil eye, i.e., using the large aperture at settings #8 focused approximately 8 cm from the right eye and giving a final magnification of approximately 3X. After UCO, varying degrees of retinal perfusion were observed, from no change in the blood flow to complete attenuation of the blood supply. In most animals, UCO had no observable effect on retinal perfusion. In many animals, a noticeable decrease in size of the blood vessels was observed, but there was no apparent blanching or whitening of the retina and no other indication of cessation of perfusion. In a small percentage of animals, UCO resulted in vessel shrinkage and obvious blanching or whitening of the retina, which indicated cessation of vascular perfusion. In Figure 1, we have attempted to illustrate what is visible through the ophthalmoscope.

The photographs were taken with a TRC-50V retinal camera (Topcon Instrument Corp. of America, Paramus, N.J.) at a final magnification of approximately 20X. Figure 1B illustrates a retina with complete attenuation of the blood supply 10 minutes after occlusion of the right carotid artery; residual venous pooling is the only observed blood presence after occlusion. However, the magnification of the retinal camera is approximately 10-fold larger than that of the Welch-Allyn scope, and only the larger arteries are visible using the Welch-Allyn scope. Only animals with a complete attenuation of the retinal blood flow (i.e., with obvious blanching of the retina) met the ophthalmoscopic selection criteria and were used for histopathologic assessment. The number of animals meeting the behavioral criteria at 1 hour after occlusion was also noted.

The animals that met the behavioral or ophthalmoscopic criteria for stroke were maintained for 3 hours with the UCO. The clamp was then removed and the blood allowed to reperfuse the brain for 24 hours. After 24 hours of reperfusion, the animals were deeply anesthetized and perfused intracardially with oxygenated Krebs-Ringer solution (pH 7.2; Sigma Chemical Co., St. Louis, Mo.) until the effluent was cleared of blood (2 minutes), followed by approximately 200 ml cold fixative for 8 minutes.

Two different histological methods were used, each using a different fixative. For glycol methacrylate resin embedding, the animals were perfused with 4% paraformaldehyde in 0.1M Sorenson's phosphate buffer, pH 7.4, plus 7% sucrose. After fixation, the brain tissue was embedded in Historesin (LKB) as previously described, 15 and 5-μm sections were cut for histological analysis. For frozen sections, the fixative consisted of 4% paraformaldehyde in 0.1M phosphate buffer, pH 7.4. Brains were postfixed for 4 hours at 4°C and equilibrated at 4°C in increasing concentrations of sucrose in phosphate-buffered saline, pH 7.4 (10%, 20%, and 30%), over a 3-day period. After complete equilibration in 30% sucrose, the brains were frozen in liquid
FIGURE 1. Photographs taken with TRC-50V retinal camera at final magnification of x20 to illustrate what is visible with Welch-Allyn Model 111 ophthalmoscope used to inspect gerbil retinas at final magnification of x3. Panel A shows fundus of normal right eye before occlusion of right common carotid artery. Note large retinal arteries (arrowheads) emerging from optic papilla. Only the larger vessels are resolved with the Welch-Allyn ophthalmoscope at settings found optimal for gerbil eye (see "Materials and Methods"). Panel B illustrates same eye 10 minutes after occlusion of right carotid artery. Arterial perfusion of retina has ceased. With Welch-Allyn ophthalmoscope, cessation of blood flow in large vessels is apparent, and blanching or whitening of the retina is obvious. Only animals demonstrating blanching of retina were accepted and processed for evaluation.

FIGURE 2. Photomicrograph of regions of brain evaluated for ischemic damage. Lines identify boundaries of CA1 region of hippocampus (H), medial cerebral cortex (MC), and lateral cerebral cortex (LC). Neuronal death was evaluated in each region using Viability Index.

Results

In the group of gerbils selected by behavioral criteria as having severe unilateral cerebral ischemia, seven of 23 (30%) met the selection criteria at 1 hour after occlusion. Histological analysis of plastic and frozen sections indicated that there was little or no tissue damage to the hemisphere contralateral to the UCO. In the ischemic hemisphere, there was large variability in the extent of ischemic damage. The Viability Index scores (Table 1) in this sample of hippocampus and lateral cortex ranged from 0 to 3; two of the seven brains showed no indication of ischemic tissue. In all brains, the medial cortex demonstrated little or no evidence of ischemic damage.

In the group of gerbils selected by ophthalmoscopic criteria as having severe unilateral cerebral ischemia, 12 of the 70 (17%) animals met the selection criteria. All 12 gerbils had severe histopathologic changes to the ipsilateral hemisphere after 24 hours of reperfusion. Interestingly, in this starting group of 70 gerbils, 19 of the animals (27%) would have met the behavioral selection criteria. Again, histological analysis indicated that there was no apparent tissue damage to the hemisphere contralateral to the UCO. In the ischemic hemisphere, the extent of postischemic neuronal loss to the hippocampus, lateral cortex, and medial cortex was more severe and consistent than that observed in the behaviorally selected group. The Viability Index scores in CA1 and lateral cortex were predominantly 3 and 4;

nitrogen vapor for 10 minutes and stored at −70°C until processing. Brains were sectioned into 50-μm slices on a Leitz sledge microtome into 0.01M phosphate-buffered saline (PBS) at 4°C. Free-floating sections were mounted out of PBS onto gelatinized slides and dried at 37°C overnight. All sections were stained with cresyl violet.

Semiquantitative histological analysis was made of the right ipsilateral hemisphere and the contralateral (noninjured) side. A Viability Index was derived for each of three brain regions as the average scores of their apparent tissue viability. Regions evaluated included the CA1 region of the hippocampus, the lateral cortex, and the medial cortex (Figure 2). Scores of 0–4 were assigned to each region of each brain section based on the percentage of neuronal loss as follows: 0, normal; 1, <20% damage (slight); 2, 20–50% damage (moderate); 3, 50–75% damage (moderate/severe); and 4, >75% damage (severe). Figures 3 and 4 show representative photomicrographs of cresyl violet–stained frozen sections as examples of the numerical scores assigned in the hippocampus (Figure 3) and lateral cortex (Figure 4). A Viability Index was derived as the mean±SEM of the scores for each region and histological preparation. Statistical evaluation was performed using one-way analysis of variance with post hoc analysis using Newman-Keul’s Multiple Range Test. The criterion set for statistical significance was p<0.05.
although less damaged, the scores for the medial cortex were also higher than those observed in the group selected by behavioral criteria. The scores for all three regions were significantly larger; i.e., the neuronal death and overall tissue damage was more severe compared with the group selected by behavioral criteria (Table 1).

Although greater cellular resolution was available in the 5-μm plastic sections, the Viability Index scores of ischemic tissue injury were essentially identical in brains embedded in plastic and in frozen brain preparations. This is especially apparent for the sample of animals selected by ophthalmoscopic criteria, in which the Viability Index scores for all three regions are identical in both the plastic and frozen sections. In the group of animals selected by behavioral criteria, the average Viability Index score of the plastic sections was apparently lower than that of the frozen sections. We believe that this difference is due primarily to the distribution of animals and not to the resolution specimen; scores of 3 were observed in the hippocampus in both plastic and frozen preparations, and there was no statistical difference between the scores of the two preparations.

Discussion

The various animal models of focal cerebral ischemia have been reviewed recently. Major advantages of the gerbil UCO model of cerebral ischemia include the simplicity of the surgery required to induce cerebral ischemia by occluding the common carotid artery, the ability to reverse the ischemia and reperfuse the brain by removal of the carotid vascular clamp, and the ability to have an internal control within the histological sample (i.e., a nonischemic and ischemic hemisphere present for comparison in the same coronal brain section). The major disadvantage of the model is the small percentage of animals that demonstrate lack of anterior collateral anastomoses and meet the selection criteria as having unilateral cerebral ischemia. In the present experiments, approximately 30% of the animals met behavioral criteria for unilateral cerebral ischemia, and only 17% reached criteria based on cessation of retinal vascular perfusion. Still, the quickness of the surgery and the ease of animal selection provide sufficient numbers of animals for experimental purposes. The restriction of the ischemia to the ipsilateral hemisphere...
in the UCO model and thus the inclusion of the contralateral nonischemic hemisphere in morphological specimens is especially useful when making enzyme histochemical or immunohistochemical analyses of potential cytoprotective agents.11

The anomalies in the gerbil cerebral vascular system, i.e., the variable anastomoses in the anterior cerebral arteries, provide the basis of the susceptibility of this animal to cerebral ischemia. Obviously, this vascular variability results in a variable degree of ischemic insult after UCO. Stereotypical behavior has been used to identify those animals that apparently have insufficient collateral anastomoses and have significant unilateral cerebral ischemia.10,11 In the present study, we found that gerbils selected by behavioral criteria still demonstrated variable degrees of ischemic insult as judged by morphometric evaluation of the lateral cortex and CA1 region of the hippocampus. In fact, approximately 30% of the animals selected by behavioral criteria did not show morphological evidence of ischemic tissue damage. This indicates that although the cerebral blood flow was reduced sufficiently to produce neurological deficits,16 sufficient blood flow was maintained through

<table>
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<tr>
<th>Region</th>
<th>Behavioral</th>
<th>Ophthalmoscopic</th>
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<tbody>
<tr>
<td>Plastic</td>
<td>n=3</td>
<td>n=6</td>
</tr>
<tr>
<td>H</td>
<td>2.7±0.3</td>
<td>3.7±0.2*</td>
</tr>
<tr>
<td>LC</td>
<td>1.3±0.3</td>
<td>3.0±0.3†</td>
</tr>
<tr>
<td>MC</td>
<td>0.3±0.3</td>
<td>1.3±0.3*</td>
</tr>
<tr>
<td>Frozen</td>
<td>n=4</td>
<td>n=6</td>
</tr>
<tr>
<td>H</td>
<td>0.8±0.8</td>
<td>3.3±0.2†</td>
</tr>
<tr>
<td>LC</td>
<td>0.8±0.5</td>
<td>3.2±0.3†</td>
</tr>
<tr>
<td>MC</td>
<td>0.3±0.3</td>
<td>1.0±0.3</td>
</tr>
<tr>
<td>All</td>
<td>n=7</td>
<td>n=12</td>
</tr>
<tr>
<td>H</td>
<td>1.6±0.6</td>
<td>3.5±0.1†</td>
</tr>
<tr>
<td>LC</td>
<td>1.0±0.3</td>
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</tr>
<tr>
<td>MC</td>
<td>0.3±0.2</td>
<td>1.2±0.2†</td>
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Values are mean±SEM; all contralateral side values=0. H, CA1 pyramidal neurons of the hippocampus; LC, lateral cortex; MC, medial cortex.

*p≤0.05, †p≤0.01.
existing collaterals to sustain the viability of the parenchyma during the 3-hour UCO and subsequent reperfusion. Thus, although behavioral evaluation is a convenient method of animal selection, the true extent of frontal brain ischemia can be variable and require larger sample groups for statistical comparisons.\textsuperscript{11} Identification of gerbils lacking anterior collateral circulation by ophthalmoscopy is technically more demanding than behavioral evaluation but offers a convenient method to accurately and reliably predict a severe uniform ischemic insult. In the group of 70 gerbils examined in these experiments, 27% met behavioral criteria, whereas only 17% met ophthalmoscopic criteria. This indicates that attenuation of retinal perfusion is a more stringent criteria for animal selection. The Viability Index of CA1, lateral cortex, and medial cortex examined in these experiments, 27% met behavioral criteria. Thus, animals selected by ophthalmoscopic criteria present a model of severe focal cerebral ischemia that minimizes the involvement of collateral anastomotic blood vessels. This model would be similar to that reported for the spontaneously hypertensive rat,\textsuperscript{17,18} in which the ischemia results in large-core necrosis, with little presence of penumbra. The present study also evaluated the advantages of two different histological preparations, i.e., 50-\(\mu\)m frozen sections and 5-\(\mu\)m plastic sections. Both methods are equally able to resolve the loss of ischemic tissue; there was not a statistically significant difference between these methods in the scoring of the ischemic tissue. Each method does provide other unique advantages. The resolution of the plastic sections is superior to that of frozen sections because of the method of embedding and the thinness of the section. In addition, because many enzymes remain active after being embedded in historesin plastic, this methodology offers an advantage for certain enzyme histochemical localizations.\textsuperscript{15} Still, there are circumstances in which the thicker frozen sections would be the method of choice. Frozen sections are generally quicker and easier to prepare, and immunohistochemical localizations are generally more successful in frozen sections than in tissues embedded in plastic. Both would provide reliable quantitative information when used consistently within a given experiment, the particular method used depending upon the specific experimental objectives.

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*Stroke*. 1992;23:1588-1593
doi: 10.1161/01.STR.23.11.1588

*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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