Evaluation of 2,3,5-Triphenyltetrazolium Chloride Staining To Delineate Rat Brain Infarcts

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Background and Purpose: Accurate and reproducible determination of the size and location of cerebral infaracts is critical for the evaluation of experimental focal cerebral ischemia. The purpose of this study was to compare intracardiac perfusion of 2,3,5-triphenyltetrazolium chloride with immersion of brain tissue in 2,3,5-triphenyltetrazolium chloride to delineate brain infarcts in rats.

Methods: After 6, 24, or 48 hours of ischemia induced by permanent middle cerebral artery occlusion, some rats were perfused with 2,3,5-triphenyltetrazolium chloride; other rats were given an overdose of barbiturates, after which brain sections were immersed in 2,3,5-triphenyltetrazolium chloride. Coronal sections were taken 4, 6, and 8 mm from the frontal pole, and infarct areas in perfused and immersed sections were compared; subsequently, the same sections were stained with hematoxylin and eosin.

Results: In rats subjected to 24 or 48 hours of occlusion, areas of infarction were clearly defined with both 2,3,5-triphenyltetrazolium chloride staining techniques, and the infarct sizes correlated well with the results of hematoxylin and eosin staining (r=0.85–0.94).

Conclusions: These results demonstrate that intracardiac perfusion of 2,3,5-triphenyltetrazolium chloride is an accurate, inexpensive, and efficient staining method to detect infarcted tissue 24 and 48 hours after the onset of ischemia in rats. (Stroke 1991;22:1394–1398)

The oxidation-reduction indicator 2,3,5-triphenyltetrazolium chloride (TTC) has been used successfully for early histochemical diagnosis of myocardial infarction. The staining action of tetrazolium salts is based on the presence of a dehydrogenase. Tissue with normal levels of the enzyme is stained red, whereas ischemic and infarcted tissue remains unstained owing to loss of the enzyme. Infarcted cerebral tissue in cats and rats can be differentiated from normal tissue by immersion in TTC solutions. Infarct size determined by this method correlates well with that determined by staining with hematoxylin and eosin (H&E). Studies in our laboratory have shown that infusion of TTC into brain by aortic injection results in better staining and clearer delineation of ischemic and nonischemic tissue than does the immersion technique. In rats subjected to permanent middle cerebral artery (MCA) occlusion, however, compromise of cerebral blood flow may prevent TTC solution from reaching the infarcted area.

To determine whether the perfusion method overestimates the size of infarcted regions compared with the immersion method or standard H&E staining, we compared infarct sizes determined by TTC perfusion or immersion with those determined by H&E staining of adjacent sections in rats subjected to permanent MCA occlusion.

Materials and Methods

Thirty-six male Sprague-Dawley rats weighing 350–400 g were divided into six groups for study. All rats underwent permanent MCA occlusion. Rats were anesthetized by intraperitoneal injection of 4% chloral hydrate solution (320 mg/kg). A curved 2-cm incision was made beginning superior to the left orbit and extending down to the left zygoma. Under an operating microscope, the temporalis muscle was reflected to expose the left MCA, the posterior part of the zygoma was resected, and a 4×4-mm craniectomy was performed just anterior to the foramen of the mandibular nerve. After the dura mater had been incised carefully with a 27-gauge needle and reflected, the MCA was occluded by microbipolar coagulation at a low power setting under contin-
uous saline irrigation. The MCA was cauterized beginning 2–3 mm medial to the olfactory tract and extending distally to the rhinal fissure. A small piece of Gelfoam was placed over the exposed brain tissue, and the temporalis muscle was laid over the craniectomy site. The skin was closed with a running 4-0 silk suture. After 6, 24, or 48 hours, each rat was reanesthetized, and TTC staining was performed as described below.

The TTC solution (2% by weight) was prepared with 37°C phosphate buffer (0.2 M Na₂HPO₄ and 0.2 M NaH₂PO₄, pH 7.4–7.6) immediately before use. Because TTC is light sensitive, solutions must be masked with aluminum foil.

For staining by the perfusion method, a blunted 18-gauge needle was passed through a small incision in the left ventricle to the proximal ascending aorta and clamped into position. The right atrium was incised, and the TTC solution was delivered by gravity flow through the needle. The rats were then wrapped in a 37°C warming blanket for 30 minutes. The brains were removed, placed in 10% buffered formalin, covered with aluminum foil, and refrigerated. Sections were photographed within 3–7 days.

For staining by the immersion method, the rat was given an overdose of barbiturate. The brain was removed, and 2-mm-thick sections were cut 4, 6, and 8 mm from the frontal pole with a customized slicer. Edematous or soft brains were placed in a freezer at −20°C for up to 20 minutes to facilitate sectioning. The sections were put in a glass Petri dish containing a shallow layer of 2% TTC, and glass coverslips wetted with the TTC solution were placed on top of each slice. To ensure even staining, the top and bottom surfaces of the section were in contact with the glass. The dishes were covered with aluminum foil to prevent exposure to light and incubated at 37°C for 30 minutes. The TTC solution was then replaced with 10% buffered formalin. To prevent distortion, brain slices were kept flat in the Petri dish overnight. The stained sections were photographed within 3–7 days.

After the TTC-stained sections had been photographed, they were stained with H&E and photographed. Tracings of coronal sections 4, 6, and 8 mm from the frontal pole and of infarcted areas were made from all three sets of photographs and quantified by an imaging analysis system (MacTablet, Planimeter software program, Reed College, Portland, Ore.). Infarcted regions from the TTC and H&E images were evaluated by persons unaware of the study groups.

The statistical significance of differences in infarct size, expressed as a percentage of hemispheric area,
between groups was determined by paired t test and linear regression analysis.

**Results**

The TTC stained normal tissue deep red; infarcted or ischemic tissue remained unstained. In tissue stained by intracardiac perfusion or immersion 24 or 48 hours after MCA occlusion, the boundary between normal and infarcted tissue was well defined and distinguishable macroscopically (Figures 1A and 2A). In rats killed at 6 hours, neither TTC method reliably differentiated between normal and infarcted tissue. The infarct areas in all groups are listed in Table 1.

In rats that underwent TTC perfusion 6 hours after MCA occlusion (group 1), ischemic or infarcted tissue not stained by TTC was poorly demarcated from the surrounding normal brain. The border zone between infarcted and normal areas was pale red. Areas of infarction determined by TTC perfusion were larger than those determined by H&E staining (23.6±1.2% versus 18.6±2.6%, p<0.05, paired t test).

In rats that underwent TTC immersion 6 hours after MCA occlusion (group 2), immersed sections were evenly stained, making it difficult to differentiate between normal and infarcted tissue. The infarct areas determined by TTC immersion did not correlate with those determined by H&E staining (2.0±0.9% versus 16.7±2.1%, p<0.005). Subsequent H&E staining showed no difference in infarct size between perfused and immersed sections (Table 1).

In rats that underwent TTC perfusion (group 3) and immersion (group 4) 24 hours after MCA occlusion, both techniques clearly defined infarcted areas, although infarct sizes were significantly larger in perfused than in immersed sections (30.4±1.5% versus 25.7±1.5%, p<0.05). H&E staining, however, showed no difference in infarct size in the two groups (Table 1). Infarct sizes determined by TTC perfusion correlated well with those determined by H&E (r=0.91, slope=0.95, y intercept=0.8%) (Figure 3). Compared with H&E staining, the perfusion method slightly overestimated and the immersion method slightly underestimated infarct size.

In rats that underwent TTC perfusion (group 5) and immersion (group 6) 48 hours after MCA occlusion, the results at 48 hours were similar to those at 24 hours. There were no differences between TTC and H&E staining, and linear regression analysis showed a signif-

![Figure 3](http://stroke.ahajournals.org/)

**Table 1. Infarct Size Determined by 2,3,5-Triphenyltetrazolium Chloride Immersion and Perfusion and by Hematoxylin and Eosin Staining**

<table>
<thead>
<tr>
<th>Group</th>
<th>Method</th>
<th>TTC</th>
<th>H&amp;E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cortex</td>
<td>Basal ganglia</td>
</tr>
<tr>
<td></td>
<td></td>
<td>plus basal ganglia</td>
<td>plus cortex</td>
</tr>
<tr>
<td>6-Hour ischemia</td>
<td>1 (n=5)</td>
<td>P</td>
<td>23.6±1.2*</td>
</tr>
<tr>
<td></td>
<td>2 (n=5)</td>
<td>I</td>
<td>2.0±0.9§</td>
</tr>
<tr>
<td>24-Hour ischemia</td>
<td>3 (n=8)</td>
<td>P</td>
<td>30.4±1.5</td>
</tr>
<tr>
<td></td>
<td>4 (n=8)</td>
<td>I</td>
<td>25.7±1.5</td>
</tr>
<tr>
<td>48-Hour ischemia</td>
<td>5 (n=5)</td>
<td>P</td>
<td>26.4±2.6</td>
</tr>
<tr>
<td></td>
<td>6 (n=5)</td>
<td>I</td>
<td>23.1±2.7</td>
</tr>
</tbody>
</table>

*Values are mean±SEM, expressed as percentage of hemispheric area. TTC, 2,3,5-triphenyltetrazolium chloride; H&E, hematoxylin and eosin; P, perfusion; I, immersion.

*p=0.01–0.05 vs. H&E.
†p<0.005 group 1 vs. group 2.
‡p<0.005 vs. H&E.
§p=0.005–0.01 vs. H&E.
¶p<0.05 group 3 vs. group 4.

FIGURE 3. Infarct size determined by 2,3,5-triphenyltetrazolium chloride (TTC) and hematoxylin and eosin (H&E) staining in each sample 24 hours after middle cerebral artery occlusion (TTC perfusion: r=0.91, slope=0.95, y intercept=0.8%; TTC immersion: r=0.85, slope=0.91, y intercept=4.5%). Dashed line, line of identity.
The larger infarct sizes may have resulted from tentatively larger and TTC immersion consistently smaller staining in each sample 48 hours after middle cerebral artery occlusion (TTC perfusion: \( r=0.92, \) slope=0.80, y intercept=4.1%; TTC immersion: \( r=0.94, \) slope=1.33, y intercept=5.3%). Dashed line, line of identity.

**Discussion**

Different methods have been used to detect the morphological features of cerebral tissue after ischemic injury.\(^7\)-\(^9\),\(^11\)-\(^13\),\(^16\)-\(^19\) One of these methods is TTC staining, which has been used to demonstrate irreversibly damaged ischemic cerebral tissue in rats\(^7\) and cats.\(^8\),\(^9\),\(^11\) In normal tissue, dehydrogenase reduces TTC to formazan, which stains red. In ischemic or infarcted tissue, however, dehydrogenase activity is reduced or eliminated\(^20\),\(^21\) and those areas remain unstained.

Consistent and reproducible results with TTC staining depend on such factors as concentration of the dye, mode of staining, and time after the ischemic insult.\(^7\)-\(^12\) Although morphological and biochemical changes in the mitochondria occur within 60 minutes after the onset of cerebral or myocardial ischemia,\(^1\),\(^4\),5,\(^10\),\(^16\),\(^17\),\(^22\)-\(^24\) the duration of ischemia is crucial for TTC staining by perfusion or immersion. In several studies, irreversibly damaged brain was detected as early as 2–3 hours after the ischemic insult,\(^10\),\(^11\) and in others only after 12–24 hours.\(^7\),\(^9\),\(^12\)

In this study, infarct sizes determined by TTC immersion, TTC perfusion, and H&E staining after 6 hours of ischemia were inconsistent. The difference between perfusion and immersion may be due to postmortem artifacts; the differences between TTC perfusion and H&E staining may be caused by mild cortical spongiosis and neuronal shrinkage in the H&E-stained sections; the difference between immersion and H&E staining may be due to inadequate or incomplete diffusion of TTC.

At 24 and 48 hours, TTC perfusion gave consistently larger and TTC immersion consistently smaller infarct sizes than those determined by H&E staining. The larger infarct sizes may have resulted from compromised accessibility of dye to all regions of the MCA after the occlusion. The smaller infarct size may be attributed to macrophage and glial cells. Glial cells surrounding damaged tissue and macrophages with intact mitochondria that infiltrate the infarcted region also contain enzymes that reduce TTC, which causes the tissue to be stained red.

The TTC perfusion technique has advantages over both TTC immersion and H&E staining. It is technically easier, less expensive, and more rapid than H&E staining; it also delineates the infarct borders more clearly than either of the other techniques. With the TTC perfusion technique, the brain is fixed after staining and is therefore easier to slice into uniform sections. For these reasons, we prefer to use the TTC perfusion technique in our laboratory.

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KEY WORDS • cerebral ischemia • histology • rats
Evaluation of 2,3,5-triphenyltetrazolium chloride staining to delineate rat brain infarcts.
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doi: 10.1161/01.STR.22.11.1394

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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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