Evaluation of 2, 3, 5-Triphenyltetrazolium Chloride as a Stain for Detection and Quantification of Experimental Cerebral Infarction in Rats

JOSHUA B. BEDERSON, M.D.,* LAWRENCE H. PITTS, M.D.,† SABELLE M. GERMANO, M.D.,* MERRY C. NISHIMURA, A.B.,* RICHARD L. DAVIS, M.D.,† AND HENRY M. BARTKOWSKI, M.D., PH.D.*

SUMMARY We have evaluated the use of 2, 3, 5-triphenyltetrazolium chloride (TTC) as an histopathologic stain for identification of infarcted rat brain tissue. The middle cerebral artery (MCA) of 35 normal adult rats was occluded surgically. At various times after surgical occlusion, rats were sacrificed and brain slices were obtained and stained with TTC or hematoxylin and eosin (H & E); the size of the area of infarcted tissue stained by each method was quantified. In rats sacrificed 24 hr after occlusion of the MCA, the size of the area of infarction was 21 ± 2% of the coronal section for TTC, and 21 ± 2% for H & E (mean ± S.D., N = 13). The size of areas of infarction determined by either staining method was not significantly different in area by the paired t test, and a significant correlation between sizes determined by each method was found by linear regression analysis (r = 0.91, slope = 0.89, and the y intercept = 4.4%). Staining with TTC is a rapid, convenient, inexpensive, and reliable method for the detection and quantification of cerebral infarction in rats 24 hr after the onset of ischemia.

THE ABILITY to determine the location and extent of areas of infarction in cerebral tissue after ischemic injury is essential for the assessment of medical or surgical interventions, for the confirmation of clinical findings, and for the evaluation of new diagnostic techniques. Traditional histopathologic methods based on staining with hematoxylin and eosin (H & E) can accurately and reliably distinguish infarcted from normal tissue, but the method is costly and time-consuming. Infarction is identified at microscopic examination by shrinkage and dark staining of neurons and swelling with vacuolization of perineurial and perivascular glial elements.1-4 The shrinkage of tissue during preparation of TTC and H & E to stain cerebral tissue in rats in area by the paired t test, and a significant correlation between sizes determined by each method was found by linear regression analysis (r = 0.91, slope = 0.89, and the y intercept = 4.4%). Staining with TTC is a rapid, convenient, inexpensive, and reliable method for the detection and quantification of cerebral infarction in rats 24 hr after the onset of ischemia.

2,3,5-Triphenyltetrazolium hydrochloride (TTC), first synthesized in 1894,6 was used originally to test the viability of seeds.7 Since 1958, TTC has been used as a stain to detect ischemic infarction in mammalian tissue.8 While this water soluble salt is not a dye, it is reduced by certain enzymes in normal tissue to a deep red, fat soluble, light-sensitive compound (formazan) that turns normal tissue deep red and thereby clearly delineates abnormal areas.7 TTC can act as a proton acceptor for many pyridine nucleotide-linked enzyme systems such as the dehydrogenases that, with the cytochromes, form an integral part of the inner mitochondrial membrane and make up the electron transport chain.7,9

TTC has been used extensively to stain myocardial tissue obtained from humans and experimental animals. TTC staining reflects accurately the extent of irreversible myocardial ischemic damage in dogs,2,10-15 and it has been reported that TTC can stain areas of injury to cerebral tissue in the cat.16,17 We report here the results of a comparative study of the use of TTC and H & E to stain cerebral tissue in rats in which infarction was produced by occlusion of the middle cerebral artery (MCA) for various times.

Methods

Thirty-five normal adult male Sprague Dawley rats weighing 300-400 gm, which were allowed free access to food and water before and after all procedures, were weighed and placed in an ether jar until they were immobilized. Anesthesia was induced with chloral hydrate dissolved in normal saline (35 mg/100 gm, intraperitoneally) and was maintained during the procedure by supplemental doses as needed. Body temperature was monitored with a rectal thermometer and was maintained within normal limits with a heating pad.

Using an operating microscope under high magnification, the left MCA was exposed transcranially18 without damage to the zygomatic bone. The brain was retracted gently to gain proximal exposure and the MCA was occluded with microbipolar forceps on a low power setting using continuous saline irrigation. Areas of infarction of different sizes were produced by occlusion of the MCA 1 to 3 mm proximal to the olfactory tract and extending to the inferior cerebral vein; the artery was transected to insure that recanal-
zation did not occur. The temporalis muscle and skin were sutured and rats were returned to cages until they either recovered from anesthesia or were sacrificed. The procedure was performed in 20 to 25 minutes; brains were retracted for less than 5 minutes.

After various survival times, rats were sacrificed by an intracardiac injection of sodium pentobarbital (75 mg of a 390 mg/cc solution). Rats were divided into four groups based on the time of sacrifice after occlusion. Time of sacrifice after occlusion for each group was: Group 1, 1 to 2 hours (N = 6); Group 2, 3 hours (N = 3); Group 3, 5 to 6 hours (N = 3); and Group 4, 24 hours (N = 23).

Within 3 min of sacrifice, brains were removed, two coronal slices were made at 5 and 7 mm from the frontal pole, and brain slices were immersed in a 2% solution of TTC in normal saline at 37°C for 30 min, after which sections were fixed in 10% phosphate-buffered formalin for photography and H & E staining.

To assess the effects of formalin and time on fading of TTC and the estimation of the size of the area of infarction, sections from 10 brains from Group 4 rats were stored in formalin for 10 to 40 days before photography and sectioning. The other specimens (N = 13) were photographed and sectioned 1 to 5 days after fixation. The rostral surface of the TTC-stained sections was photographed using color slide film (Ektachrome, tungsten 160 ASA). For comparison with H & E staining, histologic sections were prepared from the surface of the same slices and stained with H & E.

Projection tracings of the H & E and TTC stained sections from the same slice were made at different times by an independent observer who was not aware of the group from which the slices were obtained. Cortex and basal ganglia were outlined separately and the area of infarction was determined both by computer image analysis and by differential weighing of cut out areas of the tracing. Essentially identical results were obtained by both methods. The area of infarction in the cortex and the basal ganglia was expressed as the percent of the whole coronal section. Histologic sections were reviewed by one of us (R.D.) to confirm infarction size. Data for TTC and H & E sections were paired and examined for the possible existence of correlations.

Results

TTC stained normal areas of brain deep red but did not stain infarcted tissue. In tissue from Group 4 rats (24 hr after occlusion), the border between normal and infarcted tissue was well-defined and could be detected easily by gross inspection. The deep red color of TTC-stained sections disappeared during alcohol dehydration of the formalin-fixed brain slices. H & E slides prepared from these slices could not be distinguished from slices not stained with TTC, which allowed an unbiased assessment of the H & E slides. Tissue from Group 4 rats was characterized by a central area of coagulative necrosis surrounded by a zone of peripheral spongiosis. Generalized tissue pallor defined the limits of the area of infarction in each specimen. Cortex could be distinguished easily from basal ganglia within infarcted tissue (fig. 1).

For Groups 1–4, the size of the area of infarction ranged from 7.2 ± 1.5% to 21.3 ± 2.0%, determined on H & E stained sections; areas of similar size were also found on TTC stained slices. Data for the size of the area of infarction in all groups are listed in table 1.

TTC-stained tissue from Group 1 rats (1–2 h) had irregular, poorly-defined lesions that were stained pink. H & E-stained sections showed minimal changes, with small irregular areas of cortical spongiosis and neuronal shrinkage. Lesion areas determined with the two methods were not statistically different.

Areas of infarction in Group 2 rats (3 h) determined by H & E staining were twice as large as areas determined by TTC staining (14.1 ± 0.8% versus 7.0 ± 0.9%, p < 0.001), although the changes were subtle. In slices from Group 3 rats (5 to 6 h) lesions stained and identified with TTC could be seen more easily than lesions stained with H & E, and, even though sizes of areas of infarction were not significantly different, changes for H & E stained sections were still subtle.

For Group 4 rats (24 h), the size of the area of infarction was unequivocally delineated by both meth-
TABLE 1  Infarction Size Determined by TTC and H & E Staining as the Percentage of Coronal Section

<table>
<thead>
<tr>
<th>Group</th>
<th>Duration</th>
<th>TTC</th>
<th>H &amp; E</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Basal Ganglia Plus Cortex</td>
<td>Basal Ganglia</td>
</tr>
<tr>
<td>Group 4: 24 hours after occlusion</td>
<td>(N = 23)</td>
<td>21.2 ± 2.4</td>
<td>21.3 ± 2.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Basal Ganglia</td>
<td>11.8 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cortex</td>
<td>10.4 ± 1.6</td>
</tr>
<tr>
<td>Group 3: 5-6 hours after occlusion</td>
<td>(N = 3)</td>
<td>16.3 ± 5.1</td>
<td>18.0 ± 1.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Basal Ganglia</td>
<td>9.6 ± 2.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cortex</td>
<td>6.7 ± 0.7</td>
</tr>
<tr>
<td>Group 2: 3 hours after occlusion</td>
<td>(N = 3)</td>
<td>7.0 ± 0.9</td>
<td>14.1 ± 0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Basal Ganglia</td>
<td>6.3 ± 1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cortex</td>
<td>0.7 ± 0.7</td>
</tr>
<tr>
<td>Group 1: 1-2 hours after occlusion</td>
<td>(N = 6)</td>
<td>10.5 ± 1.7</td>
<td>7.2 ± 1.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Basal Ganglia</td>
<td>6.6 ± 0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cortex</td>
<td>3.9 ± 1.4</td>
</tr>
<tr>
<td>24 hours after occlusion, with delay</td>
<td>(N = 48)</td>
<td>26.0 ± 3.4</td>
<td>20.4 ± 3.6</td>
</tr>
</tbody>
</table>

Mean ± S.E.; * = p < 0.025; † = p < 0.005; ‡ = p < 0.01.
§ Ten to 40 days before photography and sectioning.

Methods, and ranged between 12.6% and 33.4% (21.3 ± 2.4%) for TTC-stained slices and 9.2% and 34.4% (21.3 ± 2.0%) for H & E-stained slices. There were no significant differences (paired t-test) within the entire lesion (basal ganglia plus cortex) and for either basal ganglia or cortex alone. Linear regression analysis showed that a significant correlation existed between data obtained for both stains (r = 0.91, slope = 0.89, y intercept = 4.4%) that did not differ statistically from a line with a slope = 1 and y intercept = 0 (H0: slope = 1 and y intercept = 0, p = 0.16, NS) (fig. 2).

Variation in the size of the area of infarction between TTC and H & E stained sections was the result primarily of the difference in the areas of infarction in the basal ganglia; despite the fact that there was no significant difference in size by the paired t-test, linear regression showed that a poor correlation (r = 0.65, slope = 0.45) existed, with a best fit line that was significantly different from slope = 1 and y intercept = 0 (p = 0.015) (fig. 3). The best correlation between the two methods was found for the measurement of the area of infarction in cortex, where r = 0.90, slope = 0.999, and y intercept = 0.60, which is not significantly different from a line with slope = 1 and y intercept = 0 (p = 0.015) (fig. 4). Thus, for Group 4 rats, TTC staining was a reliable indicator of infarction, with no false-positive or false-negative results, and could be used to quantify accurately the total size of the infarcted area compared to results obtained with H & E. However, TTC could not accurately delineate structures within infarcted tissue, and the relative contribution to the total area of infarction of lesions in the basal ganglia could not be assessed.

In photographs of TTC-stained slices from 10 Group 4 rats that were held in formalin for 10 to 40 days, color had faded visibly from the margins of normal and infarcted tissue, which made the lesion (area not stained) appear larger. For these slices, the mean size of the infarcted area was 26.1 ± 3.4% for TTC and
20.4 ± 3.6% for H & E (paired t-test; $p < 0.005$) (table 1).

Discussion

We found that it was difficult to compare results for H & E and TTC-stained sections in rats sacrificed 6 h or less after the onset of cerebral ischemia. TTC and H & E were equally effective for the determination and quantification of infarction 24 h after the onset of ischemia.

Infarction can be detected in TTC-stained infarcted coronary artery tissue in dogs as early as 1 to 2 hr after occlusion, although color differences between normal and infarcted tissue are subtle. Three to 6 hr after occlusion of the coronary artery, the border between TTC-stained normal and infarcted tissue becomes distinct and the border is delineated before the development of definite histologic evidence of infarction, and the time at which delineation occurs coincides with the rate of reduction of levels of dehydrogenase activity after occlusion of the coronary artery. Depletion of dehydrogenase is an irreversible process in myocardium and electron micrographs show that TTC reliably localizes infarcted cells only after extensive, irreversible ischemic damage has occurred. Thus, TTC-staining can detect ischemic changes in myocardium earlier than conventional methods, but by 12 to 24 h after coronary occlusion, the sensitivity of TTC and H & E are essentially equivalent and correlate significantly.

Depending on regional physiologic factors that affect cerebral tissue, ultrastructural changes may appear after 15 to 30 min of ischemia. Because the rate of cerebral oxygen consumption (CMRO$_2$) is reduced in the immediate posts ischemic period in rat brain, staining with an agent such as TTC that is based on the presence of intact enzymes of the electron transport chain might be expected to delineate infarction at an earlier stage than traditional histologic methods. However, irreversible injury to mitochondria in brain cells may not occur until after a long period of ischemia. Moreover, because coupling between electron transport and O$_2$ consumption in ischemic brain cells is altered, a decrease in CMRO$_2$, may not immediately reflect decreased dehydrogenase activity. Finally, the time necessary to cause irreversible damage to oxoreductases may be far longer than the duration of ischemia, as suggested by the results of this study. This may explain in part why unequivocal changes observed in tissue stained with TTC paralleled rather than preceded the irreversible changes seen in tissue stained with H & E.

TTC effectively and accurately detected areas of infarction 24 hr after the onset of ischemia. Because TTC can be used to stain tissue much more rapidly, easily, and cheaply than techniques based on H & E — 30 min vs. several days/rat and $\$ 0.90 vs $\$ 5.00 per rat for TTC and H & E, respectively — it is a useful method for the detection of infarction in cerebral tissue. However, it is not clear at this time whether TTC can be used for the evaluation of other types of cerebral injury. TTC staining is an excellent research method that can be used to confirm the size and location of infarction, and may be valuable to determine the presence and location of infarction in autopsy samples.

Acknowledgment

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