Effect of Brain Edema on Infarct Volume in a Focal Cerebral Ischemia Model in Rats

Teng-Nan Lin, PhD; Yong Y. He, MD; Grace Wu, MS; Myrna Khan, MS; and Chung Y. Hsu, MD, PhD

Background and Purpose: Infarct volume is one of the common indexes for assessing the extent of ischemic brain injury following focal cerebral ischemia. Accuracy in the measurement of infarct volume is compounded by posts ischemic brain edema that may increase brain volume in the infarcted region. We evaluated the effect of brain edema on infarct volume determined by triphenyltetrazolium chloride and hematoxylin and eosin stains in a focal cerebral ischemia model in rats.

Methods: In a middle cerebral artery occlusion model in rats, infarction is confined to the cerebral cortex. The infarct was delineated by triphenyltetrazolium chloride stain and, in selected samples, by hematoxylin and eosin stain. We determined infarct size at different times after the ischemic insult (6 hours to 7 days) in relation to the evolution of brain edema by the direct measurement of infarct volume. Indirect measurement to reduce the effect of edema on infarct volume was also conducted in the same brain samples.

Results: Direct measurement showed that infarct volume fluctuated with the evolution of brain edema (one-way analysis of variance, p<0.0001). Infarct volume determined by indirect measurement was independent of the extent of brain edema and remained stable from 6 hours to 3 days after ischemia. There was a good correlation between triphenyltetrazolium chloride and hematoxylin and eosin stains in delineating infarct volume with both direct and indirect measurement.

Conclusion: Traditional direct measurement of infarct volume is associated with an overestimation of infarct volume during the development of brain edema in the first 3 days after ischemia. This artifact can be reduced with indirect measurement, which is based on noninfarcted cortex volume. (Stroke 1993;24:117-121)

Key Words • brain edema • cerebral infarction • rats

Focal cerebral ischemia has been created in animals to simulate human ischemic stroke. Severe focal cerebral ischemia with or without reperfusion results in coagulation necrosis in the ischemic zone, leading to well-demarcated infarction. In animal models of focal cerebral ischemia, infarct volume is frequently determined by measuring the infarcted area in closely spaced sections of brain after special tissue staining to delineate the infarct in each brain slice.\(^1,2\) Morphometric analysis of infarct volume represents an objective and quantitative means to estimate the extent of ischemic brain injury and is a commonly employed measure to determine the efficacy of neuroprotective agents in preclinical trials.\(^3-6\) Swelling of ischemic tissue may result in enlargement of the infarcted zone, leading to overestimation of infarct volume. Brain edema has been shown to artificially increase infarct volume by as much as 22%.\(^2\) Distortion of infarct volume by brain edema may compound the interpretation of therapeutic interventions. To minimize the error that may be introduced by brain edema, Swanson et al.\(^7\) proposed a method to measure infarct volume based on the volume of surviving normal gray matter.

We have recently developed a focal cerebral ischemia model in rats that is characterized by a persistent and well-delineated infarct confined to only the cerebral cortex in the right middle cerebral artery (MCA) territory. Morphometric analysis of infarct volume was noted to be an objective measure of therapeutic interventions in this model.\(^4,6,8\) Severe brain edema evolving from 6 hours to 7 days after the ischemic insult in this model has been determined based on cortical water content in preliminary studies.\(^9,10\) This rat stroke model was employed in the present study to assess the effect of brain edema on infarct volume and to determine if the measurement of infarct volume based on surviving normal gray matter, as proposed by Swanson et al,\(^7\) is useful in minimizing the error introduced by edema.

Materials and Methods

Male Long-Evans rats weighing 250–350 g (Charles River, Wilmington, Del.) were used. Housing and anesthesia concurred with guidelines established by the institutional animal welfare committee, in accordance

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with the Public Health Service Guide for the Care and Use of Laboratory Animals, United States Department of Agriculture regulations, and the American Veterinary Medicine Association Panel on Euthanasia guidelines. The focal cerebral ischemia model has been detailed in previous publications.4,5 The rats were allowed free access to water and rat chow (Wayne, Chicago, Ill.) until surgery. Surgery leading to focal cerebral ischemia was conducted under anesthesia with 100 mg/kg i.p. ketamine and 5 mg/kg i.m. xylazine. Rectal temperature was monitored and maintained at 37±0.5°C via an electronic temperature controller (Versa-Therm 2156, Cole-Parmer Instrument Co., Chicago, Ill.) linked to a heating lamp. Physiological parameters including the plasma glucose concentration, arterial blood pressure, and pulse rate were monitored and maintained within normal ranges during surgery and ischemia as described previously.4,5 The right MCA was exposed using microsurgical techniques. Briefly, following a 2-cm vertical skin incision midway between the right eye and ear and splitting of the temporalis muscle, a 2-mm burr hole was drilled at the junction of the zygomatic arch and the squamous bone. The MCA trunk was ligated immediately above the rhinal fissure with 10-0 suture. Complete interruption of blood flow was confirmed under an operating microscope. Both common carotid arteries were then occluded using nontraumatic aneurysm clips. After the predetermined duration of ischemia (90 minutes), the aneurysm clips were removed from both common carotid arteries. Restoration of blood flow in all three arteries was observed directly under the microscope. In a separate study of the correlation between two tissue stains (see below), rats with variable durations of ischemia (30–90 minutes) were used. Free access to food and water was allowed after recovery from anesthesia. All rats were kept in air-ventilated incubators at 24±0.5°C until the end of the experiment (6 hours to 7 days).

After a predefined reperfusion period of 6 hours or 1, 3, or 7 days, the rats were killed under ketamine anesthesia by intracardiac perfusion with 200 ml of 0.9% NaCl. The brain was removed carefully and cooled in ice-cold saline for 5 minutes, then dissected into coronal 2-mm sections using a Jacobowitz brain slicer (Zivic-Miller Laboratories, Inc., Allison Park, Pa.). For the delineation of infarct area, the brain slices were incubated in isotonic phosphate-buffered saline (pH 7.4) containing 2% triphenyltetrazolium chloride (TTC) (Sigma Chemical Co., St. Louis, Mo.) at 37°C for 30 minutes and then stored in 10% neutral buffered formalin. The effect of brain edema on infarct volume has been noted previously using hematoxylin and eosin (HE) stain.6 We sought to determine if the distortion of infarct volume by brain edema was different using two different techniques, namely, TTC and HE stains. In a separate study correlating infarct volumes determined by TTC versus HE stain, brains from rats subjected to ischemia for 30–90 minutes were processed for TTC stain first. After measurement of infarcted and noninfarcted areas in the right hemisphere and of total cortex area in the left hemisphere (see below), the formalin-fixed brain slices were embedded in Lipshaw embedding matrix (Detroit, Mich.) and 20-μm slices from the middle of the 2-mm brain slices were prepared using a microtome and then stained with HE. Infarcted and noninfarcted areas in these thin slices were determined using a technique similar to that described below for TTC stain.

For morphometric measurement of infarct volume, both direct and indirect methods were employed for the same brain samples. Morphometric determination of infarct volume by the direct method has been described previously.4 Briefly, the cross-sectional area of infarction in the cerebral cortex of the right MCA territory of each brain slice was measured by a Drexel University image analyzer (DUMAS). Total infarct volume for each brain was calculated by summation of the infarcted area of all brain slices (area of infarct in square millimeters×thickness [2 mm]) from the same hemisphere. The indirect method followed a formula similar to that proposed by Swanson et al.7: RI=LT−RN, where RI=infarct volume in the right hemisphere measured by the indirect method, LT=total cortex volume in the left hemisphere of the same brain, and RN=noninfarcted cortex volume in the right hemisphere of the same brain. Total cortex volume in the right hemisphere of the same slice (RT) can be calculated as RT=RN+RI. The two formulas are valid only if LT=RT in normal brain. This contention is supported by the measurement of LT and RT in four normal rat brains (Table 1). The volumes for each category (noninfarcted cortex in the right hemisphere, total cortex in the left hemisphere, etc.) were derived from morphometric measurement of the respective category’s area in each brain slice followed by summation of areas in the same category from all slices in a brain as described above for the direct measurement of infarct volume.

The extent of postischemic brain edema was determined by the wet- and dry-weight method 6 hours to 7 days after the ischemic insult. The rats were decapitated under anesthesia. The cerebral cortex in the right MCA territory was dissected in a wet chamber saturated with water vapor.12 The cortex was then separated from the subcortical structures. The wet weight was determined immediately. The dry weight was determined after drying the tissue to a constant weight at 100°C. Tissue water content was calculated as % $H_2O=(1−\text{dry wt/wet wt})\times100%$.13,14

**Statistical Analysis**

To compare multiple means of infarct volume and water content, statistical analysis was performed using one-way analysis of variance. The level of significance for a difference between two groups was further analyzed with post-hoc Tukey’s protected $t$ tests using statistical software (GB-STAT 2.1, Dynamic Microsystems, Inc., Silver Springs, Md.). A probability value of less than 0.05 was considered to be significant. To determine the relation between infarct areas and volumes determined by the two different stains (TTC and HE), linear regression was employed. Infarct area or volume based on TTC staining was regressed on that determined by HE staining. Equality of slopes and intercepts of the least-square lines were tested according to Snedecor and Cochran.15 Differences in infarct areas and volumes defined by TTC and HE stains and measured by the direct and indirect methods, respectively, were also assessed by independent $t$ tests using the SAS statistical package (SAS Institute, Inc., Cary, N.C.).
Table 1. Chronological Study of Brain Infarct Volume After Focal Ischemia in Rats

<table>
<thead>
<tr>
<th>Time after ischemia</th>
<th>Infarct volume (mm³)</th>
<th>Total cortex volume (mm³)</th>
<th>Noninfarct cortex volume in right hemisphere (mm³)</th>
<th>Infarct volume in right hemisphere (indirect measurement) (mm³)</th>
<th>Direct/indirect infarct volume ratio</th>
<th>% H₂O in right cortex</th>
</tr>
</thead>
<tbody>
<tr>
<td>No ischemia</td>
<td>4</td>
<td>0</td>
<td>400.4±16.4</td>
<td>397.7±13.2*</td>
<td>.</td>
<td>78.60±0.12*</td>
</tr>
<tr>
<td>90 min ischemia</td>
<td>6</td>
<td>0</td>
<td>182.5±12.2†</td>
<td>395.0±16.4</td>
<td>.</td>
<td>84.45±0.17*</td>
</tr>
<tr>
<td>6 hr</td>
<td>8</td>
<td>0</td>
<td>228.6±15.4</td>
<td>412.5±6.2</td>
<td>.</td>
<td>89.41±0.24</td>
</tr>
<tr>
<td>24 hr</td>
<td>6</td>
<td>0</td>
<td>185.1±9.1†</td>
<td>418.4±19.6</td>
<td>.</td>
<td>85.30±0.50*</td>
</tr>
<tr>
<td>72 hr</td>
<td>7</td>
<td>0</td>
<td>92.5±11.0*</td>
<td>382.9±18.3</td>
<td>.</td>
<td>83.01±0.36*</td>
</tr>
<tr>
<td>1 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>.</td>
<td>80.07±0.01</td>
</tr>
<tr>
<td>p</td>
<td>.</td>
<td>.</td>
<td>&lt;0.0001</td>
<td>.</td>
<td>.</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Values are mean±SEM; p reflecting significance level of difference among groups was calculated for each parameter (e.g., infarct volume by direct measurements, % H₂O, etc.) using one-way analysis of variance. Because edema peaked at 24 hours after ischemia, difference of other time points from 24-hour group was also determined.

*p<0.01, †p<0.05 different from 24-hour group by post-hoc Tukey’s protected t test.

Results

Table 1 shows the evolution of brain edema based on water content in the right MCA cortex. Brain edema appeared to peak at 24 hours postischemia. Differences in water content were significant among different time points. Table 1 also shows infarct volumes in the right hemisphere at various times after ischemia/reperfusion. Infarct volume determined by direct measurement was greatest 24 hours after the ischemic insult and was smallest at 1 week. Infarct volume based on direct measurement thus appeared to fluctuate over time in a pattern corresponding to the evolution of brain edema during the same period (Table 1). Changes in infarct volume over time were significant. Infarct volumes based on indirect measurement using the formula proposed by Swanson et al is also shown in Table 1. Infarct volume determined by indirect measurement remained constant from 6 hours to 3 days after the ischemic insult. Infarct volume based on indirect measurement at 1 week after ischemia appeared smaller than volumes determined 6 hours to 3 days after ischemia. Infarct volumes determined by indirect measurement were not different among time groups. There were also time-dependent changes in the total cortex volume in the right hemisphere that appeared to fluctuate with the evolution of brain edema; a significant difference was noted. In contrast, changes in total cortex volume in the left hemisphere were minimal and not significant (Table 1).

To further determine if the effect of brain edema on infarct volume differed between TTC and HE stains, we also compared infarct volumes determined by these two methods. Systematic study of the effect of brain edema on infarct volume by HE staining unfortunately was not possible because fragility of aged (>1 day) infarct prevents direct measurement of infarct area in all brain slices. However, random thin brain slices were successfully prepared from slices with infarct aged 1 or 3 days. Infarcts from these slices measured by HE stain could be compared with corresponding infarct areas measured by TTC stain. There was a close relationship between stains in delineating infarct area in these brain slices determined by both direct and indirect measurement (Figures 1 and 2). The correlations of infarct area between TTC and HE stains were similar for brain slices prepared from 1- and 3-day-old infarcts. In a few brains with all slices successfully prepared for HE stain, infarct volumes determined by HE stain were highly correlated with those determined by TTC stain using both direct and indirect measurement (Figures 3 and 4). Linear regression revealed no significant difference in the slopes and intercepts of the least-square lines for TTC versus HE stains for 1- or 3-day-old infarcts.

Discussion

Results from this study show a wide fluctuation of infarct volume determined by direct measurement that appears to vary with the evolution of brain edema. This finding confirms earlier reports2,7 that edema may introduce an artifact into the measurement of infarct volume. The smaller fluctuation of infarct volume based on a formula proposed by Swanson et al supports their

Figure 1. Scatterplot of relation between direct measurement of infarct area in mm² stained by triphenyltetrazolium chloride (TTC) and hematoxylin and eosin (H&E) in 61 prospective slices with infarction. □, 1-day-old slices; ■, 3-day-old slices. Y = 1.85 + 0.84X, r = 0.845, p = 0.0001. Differences between areas stained by H&E and TTC were compared between 1- and 3-day-old slices by independent t test (t = -1.84, df = 59, p = 0.07).
contention that indirect measurement of infarct volume based on the noninfarcted volume is useful in reducing the time-dependent variation of infarct volume that may be caused by edema. Indirect measurement results in almost constant infarct volume between 6 hours and 3 days after the ischemic insult, virtually eliminating the changes in infarct volume over time that were noted with direct measurement. Because fluctuation of infarct volume by direct measurement appeared to correspond with the evolution of brain edema, it is likely that brain edema leading to swelling of the infarcted cortex contributed at least partially to the time-dependent fluctuation in infarct volume. To eliminate overestimation of infarct volume during a period when brain edema is prominent, indirect measurement using the formula proposed by Swanson et al\textsuperscript{7} offers a desirable alternative.

We have previously shown a close correlation of infarct volumes determined by TTC and HE stains plus Luxol fast blue in 1-day-old infarcts.\textsuperscript{4} Because of technical difficulty in preparing thin brain slices from aged infarct, a chronological study of the effect of brain edema on infarct volume determined by HE stain was not possible beyond the first day. Nevertheless, TTC and HE stains showed good correlation in delineating infarct areas in some slices successfully prepared from 3-day-old brains. These findings suggest that the effect of edema on infarct volume probably occurs with TTC as well as with HE stain. The effect of edema on infarct volume has been previously shown using HE stain.\textsuperscript{2}

We noted a trend for cortex volume to be smaller in both hemispheres 1 week after the ischemic insult, suggesting resolution of brain swelling and possible loss of brain tissue after ischemia. This was significant in the right but not the left hemisphere. There was also a trend for the noninfarcted cortex volume in the right hemisphere to be greater 1 week after ischemia than earlier. This could be related to active gliosis, macrophage infiltration, and other proliferative processes involving vascular tissue in the periphery of the infarct that exhibit intense TTC staining.\textsuperscript{16} This may potentially be an artifact leading to underestimation of infarct volume 1 week after ischemia. This underestimation was apparently greater with direct than with indirect measurement. The indirect measurement proposed by Swanson and coworkers\textsuperscript{7} is based on the assumption that edema does not occur outside the infarcted region (in the ipsilateral noninfarcted cortex and the contralateral hemisphere). The lack of significant differences in cortex volumes over time in these two regions suggests that potential errors secondary to edema-induced changes of noninfarcted cortex are minimal.

In conclusion, a systematic study of the evolution of brain edema and infarct volume shows that infarct volume could be overestimated by direct measurement at the time of peak brain edema. This artifact could be substantially reduced by an indirect measurement.
method recommended by Swanson et al. Limited studies of infarct volume using HE stain suggest that the effect of brain edema on infarct volume occurs with HE as well as with TTC stain.

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References


Editorial Comment

These studies test the accuracy of two methods, direct and indirect, for determining infarct volume associated with a focal cerebral ischemic model. The experiments compared tissue using triphenyltetrazolium chloride (TTC) and conventional morphometric measures based on hematoxylin and eosin (H&E)-stained material. They concluded that the indirect method introduced earlier by Swanson et al. was the most accurate, as infarct volume was seen not to vary as edema developed over the days after infarction.

In both the current studies and the studies by Swanson et al., an image processor was used to assess noninfarcted cortex, with thresholds based on the uninjured hemisphere. The message is clear. Studies of infarct volumes using direct measures will be in error when edema is present. This was confirmed in the present study by measuring water content using conventional wet/dry techniques. Interestingly, one can estimate the increase in tissue volume by the difference in tissue percent water. According to the data of this article, the percent water of the right middle cerebral artery cortex increased from 78.6% to 89.41% by 24 hours (Table 1 of Lin et al.). This corresponds to a volume increase of 13.75% as calculated by the equation dV = swelling volume % = ([(89.41 – 78.6)/78.6] x 100.

During the same time interval, the right cortex volume (in cubic millimeters) measured morphometrically increased from a mean level of 397.7 to 471.5; this represented an increase of 18.5%, which is reasonably close to swelling caused by the increased edema. Thus, one can expect an artifactual increase in infarct volume of this magnitude, as emphasized by Lin and coworkers. The studies by this group are of major importance in clarifying the underlying pathophysiology of ischemic brain injury, particularly in assessment of treatment requiring an accurate assessment of infarct volume. This confirmation with both the TTC and H&E methods coupled with the earlier studies by Swanson would indicate that the indirect method is clearly most appropriate for investigations of ischemia.

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