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A Brief Review of Edema-Adjusted Infarct Volume Measurement Techniques for Rodent Focal Cerebral Ischemia Models with Practical Recommendations

Cyrus Nouraee[1], **Marc Fisher**[2], **Mario Di Napoli**[3], **Pascal Salazar**[4], **Tracy D. Farr**[5], **Alibay Jafarli**[1], and **Afshin A. Divani**[1,6,7*]

[1]*Department of Neurology, University of Minnesota, Minneapolis, MN, USA*

[2]*Department of Neurology, Beth Israel Lahey Health, Harvard Medical School, Boston, MA, USA*

[3]*Department of Neurology, San Camillo de' Lellis District General Hospital, Rieti, Italy*

[4]*Vital Images, Minnetonka, MN, USA*

[5]*School of Life Sciences, Faculty of Medicine and Health Sciences, University of Nottingham, Nottingham, UK*

[6]*Department of Neurosurgery, University of Minnesota, Minneapolis, MN, USA*

[7]*Department of Biomedical Engineering, University of Minnesota, Minneapolis, MN, USA*

Abstract

Background—Determining cerebral infarction volume is an important part of preclinical studies to determine the benefit of potential therapies on stroke outcome. A well-known problem in determining the actual infarction volume of rodent models is the presence of edema. Because of this, algorithms must be utilized to obtain the edema-adjusted (E_A)-infarct volume. Different methods based on 2,3,5-triphenyltetrazolium hydrochloride (TTC) staining have been published describing algorithms to determine the E_A-infarct volume.

Materials and Methods—Simulated models of infarction and corresponding swelling were employed to determine which absolute method of calculation (Lin et al., Reglodi et al., or Belayev et al.) is the most accurate in calculating the absolute E_A-infarct volume.

Results—The Reglodi and Belayev methods were statistically more accurate in measuring E_A-infarct volume than Lin's method, $p = 0.0078$. Though there was no significant difference between Reglodi's and Belayev's methods for the E_A-infarction volume calculation, Reglodi's approach was closer to the ground-truth infarct volume while also being simpler and more straightforward to use.

Conclusion—We recommend that Reglodi's method, that is E_A-infarct volume = infarct volume × (contralateral hemisphere/ipsilateral hemisphere), to be used in calculating E_A-infarct volume in TTC stained rodent brains. Further, factors such as inhomogeneous infarction distribution in a given brain slice can also contribute to the error in volume calculation. Therefore, the average of the infarct area obtained from anterior and posterior views of a given slice should be used to account for the variation. Considering different factors, we have provided a summary recommendation for calculating the infarction volume.

Keywords

Stroke model; rodents; tetrazolium salt; focal cerebral ischemia; cerebral infarction measurement; 2,3,5-triphenyltetrazolium hydrochloride (TTC)

INTRODUCTION

Every year, approximately 795,000 people experience a stroke, with 87% of all strokes being ischemic strokes [1]. In order to study stroke and potential therapies, rat models are used to model stroke in humans due to the

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***Corresponding Author:** Afshin A. Divani PhD, Department of Neurology, University of Minnesota, MMC 295, 420 Delaware Street S.E., Minneapolis, MN 55455, USA. adivani@gmail.com.

similar physiology and cerebral vasculature between the two species [2]. Additionally, rat models are useful in studies due to the ease of conducting reproducible studies [3]. Cerebral infarction volume is indicative of stroke severity and related to neurological deficits [4]. Therefore, determining infarcted brain tissue volume is an important part of preclinical studies to determine the beneficial effects of potential therapies on stroke outcome [5,6]. A well-known problem in determining the actual infarction volume of rodent brains that have undergone middle cerebral artery occlusion (MCAo) is the presence of edema during the acute and subacute phases of ischemic injury [7]. The presence of edema causes swelling of the brain tissue leading to an overestimation of the actual brain infarction volume, making a direct measurement of the infarction volume relatively inaccurate. Consequently, algorithms must be utilized to adjust for the presence of edema when determining infarction volume, during the acute and subacute stages poststroke.

One of the most common methods to visualize cerebral infarction volume in rodent MCAo models is 2,3,5-triphenyltetrazolium hydrochloride (TTC) staining [8]. Different methods by which adjusted infarction volume can be measured via TTC staining have been described [9–14]. In this paper, we compare the different measurement algorithms and provide practical recommendations by simulating scenarios with different infraction sizes and swelling amounts to compare the accuracy of the published algorithms.

BRIEF REVIEW OF PUBLISHED ALGORITHMS FOR MEASURING EDEMA-ADJUSTED BRAIN INFARCT SIZE

The main difference in the suggested algorithms [9–14] for edema-adjusted (E_A)-infarct volume calculation is based on the assumption of whether the edema is contained within the infarcted tissue region or has spread into the non-infarcted tissue of the ipsilateral hemisphere. Further, some present the E_A-infarct volume calculation in a dimensional analysis such as a ratio or percent difference, while others present it as an absolute measure.

Swanson et al. [14] introduced a method that takes into account the percent difference between the brain contralateral hemisphere volume and that of the non-infarcted region of the ipsilateral hemisphere to arrive at the percent E_A-infarct volume (dimensional analysis). Lin et al. [11] used Swanson et al.'s approach by taking the difference in volume between the contralateral hemisphere and the non-infarcted region of the ipsilateral hemisphere to arrive at an absolute volume of the infarct tissue. Freret et al. [10] described an algorithm similar to the one proposed by Lin et al., with slight modifications that take the difference between the entire contralateral hemisphere and the non-infarcted region of the ipsilateral hemisphere, dividing it by the area of the structures in the contralateral hemisphere (dimensional analysis). Chelluboina et al. [9] have also employed a method that is similar to the one proposed by Freret et al. [10]. The final value represents the proportion or percent difference of the infarcted tissue to that of the contralateral hemisphere. All of the aforementioned methods assume the absence of edema in the ipsilateral non-infarcted region.

Table 1. The various published methods, with algorithms, for calculating the non-edema infarction volume

Author	Algorithm	Quantification
Swanson et al. [14]	$I_{EA} = (L - N)/L*100$	Percent infarct volume
Freret et al. [10]	$I_{EA} = (L - N)/L$	Infarct volume ratio
Lin et al. [11]	$I_{EA} = L - N$	Absolute infarct volume
Reglodi et al. [13]	$I_{EA} = I*(L/R)$	Absolute infarct volume
Belayev et al. [16]	$I_{EA} = I*(1 - [(R - L)/L])$	Absolute infarct volume

I: absolute unadjusted infarct area; I_{EA}: edema-adjusted *I; L*: left (contralateral) hemisphere area; *R*: right (ipsilateral) hemisphere area; *N*: non-infarcted tissue in the ipsilateral region.

Note: only the absolute infarct volume algorithms are compared with each other in this study.

Some studies have shown that focal cerebral ischemia in rat models produces an enlargement of the entire ipsilateral hemisphere with a potential midline shift [15]. Based on this assumption, Reglodi et al. [13] discuss a method that takes a ratio of the volume of the contralateral and ipsilateral hemispheres as a scaling factor (dimensionless) to adjust the measured infarct volume for the presence of edema. McBride et al. [12] simply present the same algorithm that was previously described by Reglodi et al. [13] but used the method proposed by Lin et al. [11] to indirectly measure the infarct volume. Similar to the Reglodi et al. [13] approach, Belayev et al. [16] proposed the use of a scaling factor to adjust the directly measured infarct volume. Assuming a scaling factor with a range of 0–10, they have subtracted the ratio of the difference in size between the hemispheres over the contralateral hemisphere from 1. These methods take into account the presence of edema in the entire ipsilateral hemisphere and not just in the infarcted tissue. Table 1 summarizes these algorithms,

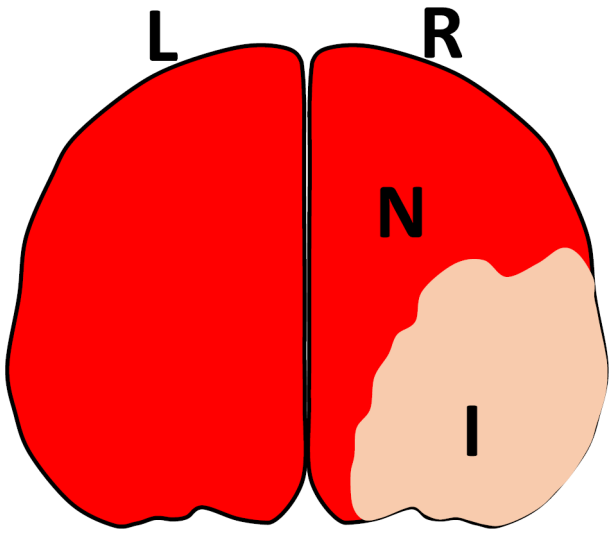

Figure 1. A simulated focal cerebral ischemia in the lower right region of the right hemisphere of the brain. *L* is the area of the entire contralateral hemisphere, *R* is the area of the entire ipsilateral hemisphere, *N* is the area of the non-infarcted region in the ipsilateral hemisphere, and *I* is the area of the infarcted region of the ipsilateral hemisphere.

and Figure 1 shows the regions and their corresponding labels that are used for the various algorithms.

MATERIALS AND METHODS

For simplicity, we have assumed that the infarct tissue is limited to one 2-mm brain slice. Therefore, the calculations presented here are for a single brain slice sample to assess the difference between the obtained results. In a real case scenario, the total infarction volume is simply a summation of the infarction volumes observed in each slice of the brain. We performed calculations only for the algorithms in Table 1 that offer absolute infarct volume calculations (i.e., Lin et al. [11], Reglodi et al. [13], and Belayev et al. [16]). We have used a common vernacular to describe all three methods of calculation. To compare the discussed three algorithms and their accuracy in measuring E_A-infarct volume, we used eight brain infarction simulations (see Figure 2 for a representative sample) with similar brain sizes to that of adult rats. Samples A and B in Figure 2 are non-edema infarcted brain tissues with different random infarction patterns (ground-truth references). Samples A1 and A2 correspond to randomly chosen different amounts of swelling in the ipsilateral hemisphere for sample A, while samples B1 and B2 similarly correspond to sample B with differing amounts of swelling in the ipsilateral hemisphere. Image J (NIH) was used to calculate the areas of different regions (including the infarction areas) calibrated based on the pixel size in the image. The volumes were calculated by multiplying the measured areas by a slice thickness that is commonly used in laboratory practice (2 mm).

STATISTICAL ANALYSIS

The differences between absolute infarction volumes of each method and the reference volume were tested using Wilcoxon tests for paired samples (two-tailed). For these tests, *p*-values were corrected for family-wise error using the Holm–Bonferroni method. Moreover, the absolute relative differences between measured volume and reference volume were computed as absolute value [(Vol. Measured − Vol. Reference)/Vol. Reference] and their differences were tested using the same Wilcoxon tests. The significance level value was set to <0.05.

RESULTS

The mean reference infarction volume was 29.77 mm^3 (95%CI: [26.56, 32.98]). The mean infarction volume using Reglodi's method was 30.15 mm^3 (95%CI: [27.1, 33.17]), using Belayev's method was 28.29 mm^3 (95%CI: [25.70, 30.70]), and using Lin's method was 19.94 mm^3 (95%CI: [17.02, 22.86]). Volumes obtained from Lin's method were significantly different (smaller) from the reference volumes ($p = 0.048$), from Reglodi's method volumes ($p = 0.048$) and from Belayev's method volumes ($p = 0.048$). Moreover, Belayev's method volumes were also significantly smaller than Reglodi's method volumes ($p = 0.048$), see Figure 3.

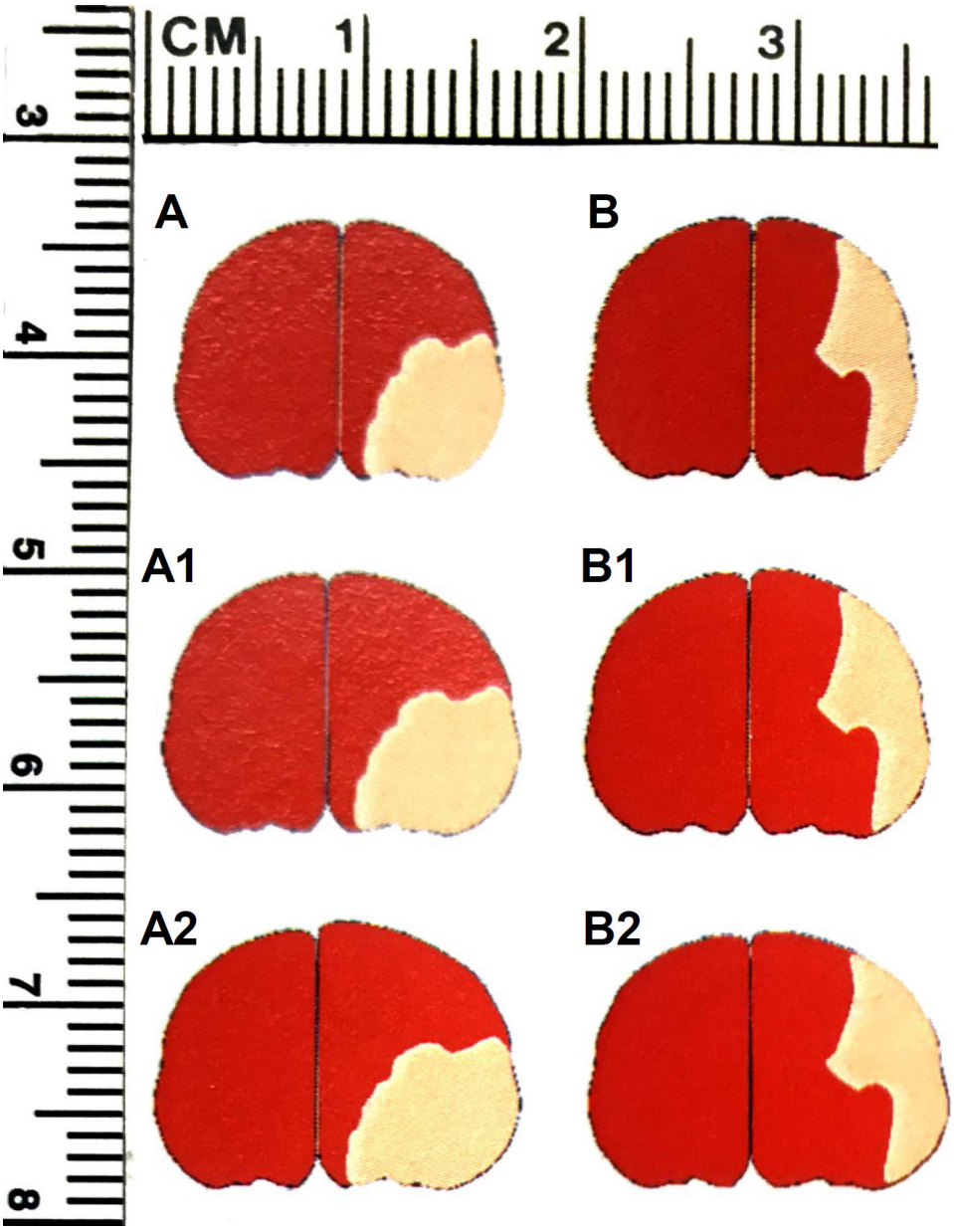

Figure 2. Simulations of varying sizes of infarction and edema. Simulation A and B are infarction sizes and shapes in non-edema brain tissue. Simulations A1 and A2 correspond to infarction A with two random degrees of edema in the ipsilateral hemisphere. Simulations B1 and B2 correspond to infarction B with two random degrees of edema in the ipsilateral hemisphere.

The volumes obtained from Reglodi's (p = 0.461) and Belayev's (p = 0.109) methods were not significantly different from the reference volumes. However, this should be interpreted with caution knowing the small sample size (n = 8), see Figure 3.

The mean ± SD of relative volume differences were 2.76% ± 2.51, 5.89% ± 4.40, and 32.98% ± 8.53 for Reglodi's, Belayev's, and Lin's methods, respectively. Both Reglodi's and Belayev's methods were significantly different from Lin's method (p = 0.0078).

DISCUSSION

We compared the three absolute E_A-infarct volume calculation methods (i.e., Lin et al. [11], Reglodi et al. [13],

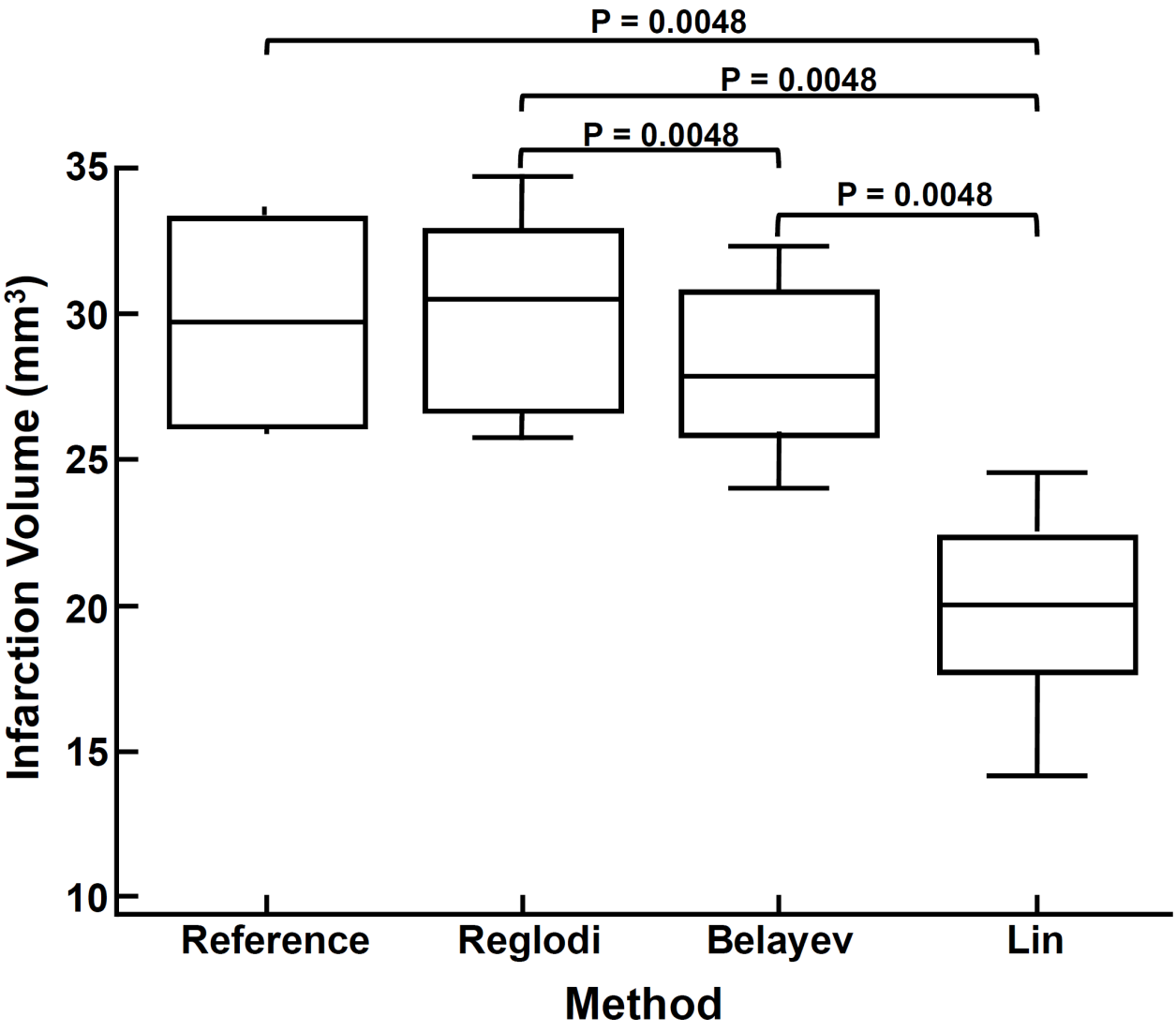

Figure 3. Measured infarction volumes using three different algorithms and the reference volumes.

and Belayev et al. [16]) here. In this study, simulated models of infarction and corresponding swelling were employed to determine which method is the most accurate in calculating the absolute E_A-infarct volume.

Cerebral edema regularly accompanies ischemic cerebral infarction and is composed of cytotoxic and vasogenic edema [7]. Cytotoxic edema evolves over minutes to hours and may be reversible, while the vasogenic phase occurs over hours to days, and is considered an irreversibly damaging process [7]. MRI studies of a rat MCAo model revealed a direct association between the size of the induced infarction and the amount of edema that peaks by 24 hours postinjury, where the estimated brain tissue swelling at 24 hours was about 24.5% of the total infarcted volume [17]. Since vasogenic edema extends well beyond the infarcted tissue region [15,18], we believe the E_A-infarction volume should be based on an algorithm that assumes that there is edema in the non-infarct ipsilateral region. This is supported by the significantly lower E_A-infarction volume using Lin's method compared to the reference volume (p = 0.0048) and compared to either Reglodi and Belayev's methods volumes (both p = 0.0048). From this, we can conclude that both the Reglodi's and Belayev's algorithms, both with a direct measurement of the infarct area, are significantly better at measuring the E_A-infarction size than the Lin algorithm.

Additionally, though Belayev's method volumes were not significantly different from the reference volumes (p

= 0.109), Reglodi's E_A-infarction size calculation was closer to the true infarct size (p = 0.461) and a significant difference was found between Reglodi's method volumes and Belayev's method volumes (p = 0.0048). Further, the algorithm proposed by Reglodi et al. is simpler and more straightforward to use, and it is, therefore, recommended.

It is worthwhile to consider that the role and amount of edema in aggravating the primary brain ischemic injury should also be investigated separately in-vivo using longitudinal MRI studies. Further, ex-vivo assessment of water content, using a wet/dry method [19], should be performed by measuring the water content of the tissue samples from the non-infarct and infarct regions of the ipsilateral hemisphere and their corresponding contralateral regions. However, the difference between in-vivo and ex-vivo studies can be complementary when the observed differences are assessed vis-a-vis brain tissue compression due to swelling in the presence of a space-limiting skull that can lead to inward swelling, comparison of the ventricles, midline shift, and consequently compression of the contralateral hemisphere [20]. The intracranial pressure causing inward deformation of the brain tissue is not present when it is harvested for TTC staining.

It is also important to note that while a proper algorithm for removing the effect of swelling when the infarcted tissue is measured plays an important role, other factors contributing to the accuracy of the measurement should also be considered. For example, proper handling of the fresh tissue for TTC staining should be considered to minimize damage to the tissue that can affect the measurement. Further, having a homogenous distribution of the infarction in a given brain slice (see Figure 4) may not be a correct assumption and can add significant error to the measurement. Therefore, each slice should be photographed from both the anterior and posterior views, and the average of the infarction area from each view should be considered to be multiplied by the slice thickness. Also, when measuring the area of the contralateral or ipsilateral brain hemisphere, the variation in the area observed in the anterior and posterior views due to the curvature of the brain (see Figure 5) should be considered. Thus, the area of each hemisphere should also be the average of the areas measured from each view.

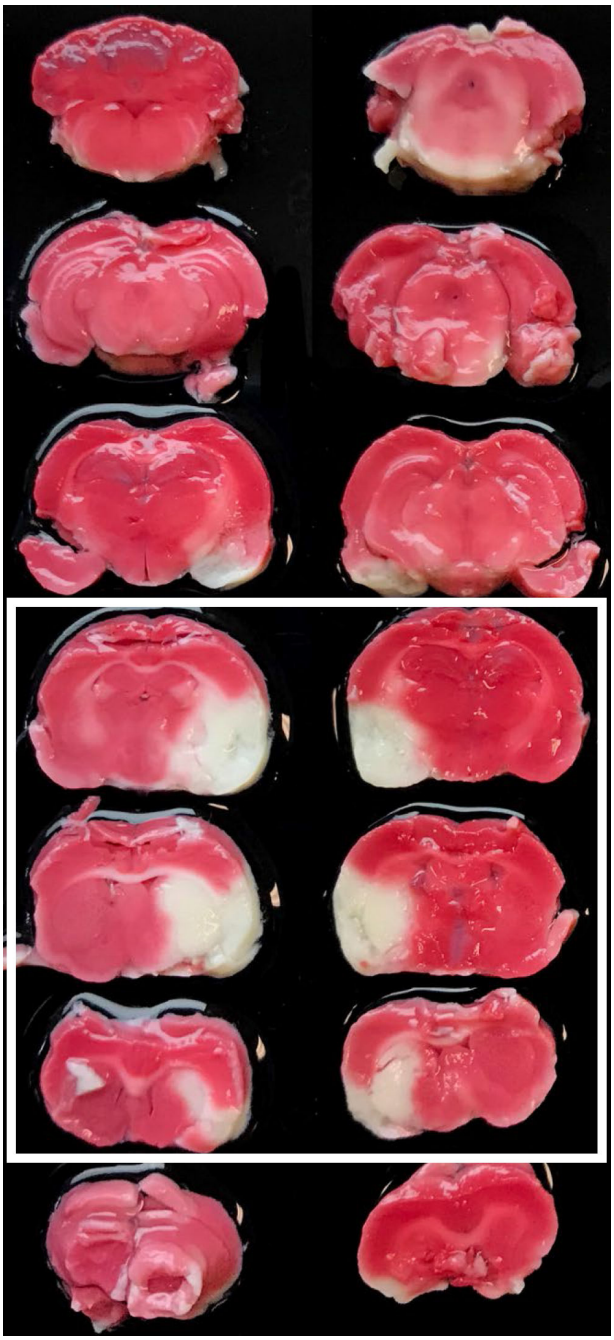

Figure 4. Anterior (left column) and posterior (right column) views of a sample of rat brain infarction after MCAo. The slices are cut 2 mm thick. There is a distinct difference in the area of infarction between the anterior and posterior views (particularly for those shown in the box).

RECOMMENDATIONS FOR INFARCTION VOLUME CALCULATIONS

- Photograph the brain slices from both the anterior and posterior views by having two plastic rulers placed vertically and horizontally next to the brain slices.

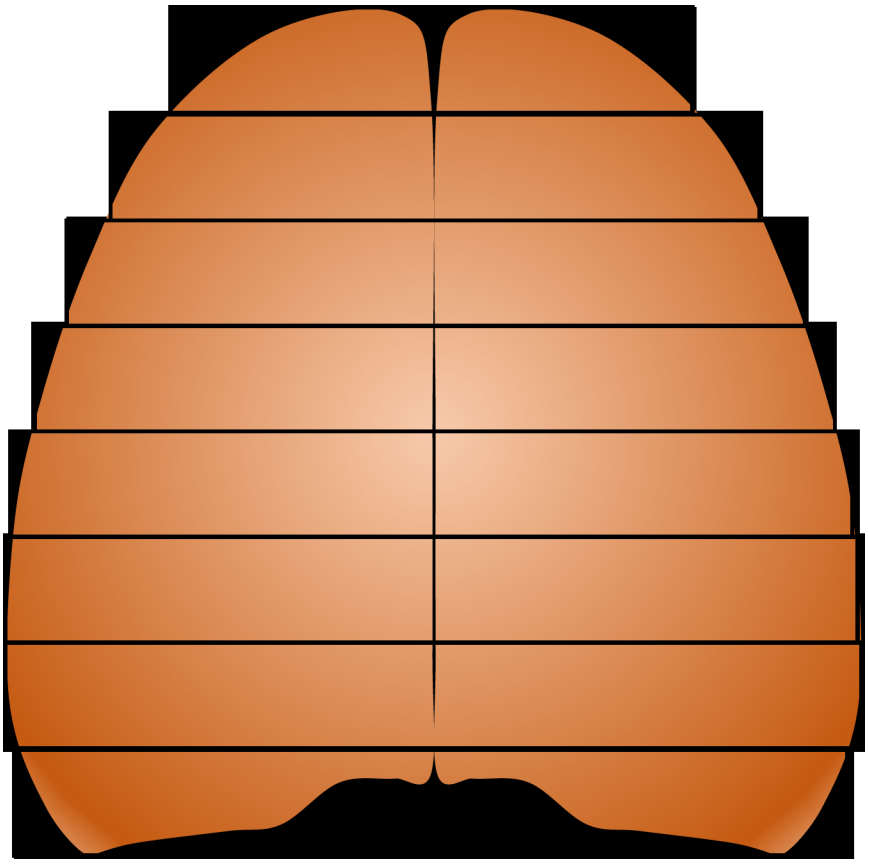

Figure 5. A schematic of a rat brain shape with 2-mm thick cuts. The black boxes delineate 2-mm cut blocks.

- Since the infarction may not develop homogenously throughout the slice thickness (see Figure 4), the infarct volume measured for each brain slice should be the average infarct areas observed on the anterior and posterior views.
- Due to the curved nature of the brain (see Figure 5), the ipsilateral and contralateral brain hemispheres area/volume should be obtained by averaging the values obtained from both the anterior and posterior views.
- The measured infarct area for each slice should be adjusted for edema by multiplying the measured value by the scaling factor C/I (area of contralateral hemisphere/area of ipsilateral hemisphere).
- The volume of E_A-infarct tissue can be obtained by multiplying it by the slice thickness (usually 2 mm).
- The total absolute infarct volume is the sum of infarct volumes calculated for each slice.
- The ratio of the infarct volume to the brain hemisphere can be obtained by dividing the infarct volume by the contralateral hemisphere volume (or 2 × contralateral volume for the ratio with respect to the whole brain volume).
- During measurment, if there is missing tissue due to the damage from infarction or problem with proper cutting, it is advised to draw an imaginary line using the contralateral side as a reference.
- When outlining the boundaries of the hemispheres, it is important to select the boundary corresponding to the anterior or posterior views to minimize error in calculating the area of each hemisphere. The area of each hemisphere for a given slice is then the average of the two measurements obtained from the anterior and posterior views.

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