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TTC, Fluoro-Jade B and NeuN staining confirm evolving phases of infarction induced by middle cerebral artery occlusion

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ABSTRACT

Considerable debate exists in the literature on how best to measure infarct damage and at what point after middle cerebral artery occlusion (MCAO) infarct is histologically complete. As many researchers are focusing on more chronic endpoints in neuroprotection studies it is important to evaluate histological damage at later time points to ensure that standard methods of tissue injury measurement are accurate. To compare tissue viability at both acute and sub-acute time points, we used 2,3,5-triphenyltetrazolium chloride (TTC), Fluoro-Jade B, and NeuN staining to examine the evolving phases of infarction induced by a 90-min MCAO in mice. Stroke outcomes were examined at 1.5 h, 6 h, 12 h, 24 h, 3 d, and 7 d after MCAO. There was a time-dependent increase in infarct volume from 1.5 h to 24 h in the cortex, followed by a plateau from 24 h to 7 d after stroke. Striatal infarcts were complete by 12 h. Fluoro-Jade B staining peaked at 24 h and was minimal by 7 d. Our results indicated that histological damage as measured by TTC and Fluoro-Jade B reaches its peak by 24 h after stroke in a reperfusion model of MCAO in mice. TTC staining can be accurately performed as late as 7 d after stroke. Neurological deficits do not correlate with the structural lesion but rather transient impairment of function. As the infarct is complete by 24 h and even earlier in the striatum, even the most efficacious neuroprotective therapies are unlikely to show any efficacy if given after this point.

1. Introduction

Experimental stroke models are essential to study the pathophysiology of cerebral ischemia and to evaluate the effects of novel therapeutic interventions. The MCAO model in rodents has been widely used to study focal cerebral ischemia. This model offers a simpler and less traumatic surgical approach compared with earlier craniotomy models (Tamura et al., 1981), lends itself more readily to the study of reperfusion and has been adapted for use in continuous magnetic resonance imaging (Roussel et al., 1995). However significant controversy exists due to the variability of final infarct size and debate as to the most reliable time point to measure the effects of various therapeutic agents (DeVries et al., 2001; Culmsee et al., 2005; Hoyte et al., 2006). Although transient MCAO model has been utilized to study ischemic stroke for decades, the evolution of infarct within the area blood supplied by MCA has not been well elucidated. In the present study we performed an analysis to investigate the evolution of infarct after MCAO. We performed this analysis in mice, a model system that has been less well characterized (Duckworth et al., 2005).

In order to analyze the time-dependent changes following transient MCAO, several different histochemical methodologies can be utilized. 2,3,5-Triphenyltetrazolium chloride (TTC) is one of the most common histochemical stains used to assess cerebral injury. In ischemic tissue, lack of TTC staining is considered "infarcted" and defined as core and viable tissue is stained red (Benedek et al., 2006). Although widely accepted and used, TTC staining has received criticism as TTC is a marker of tissue dehydrogenase and mitochondrial dysfunction and may not represent irreversible cell death, therefore this method may overestimate infarct size (Tureyen et al., 2004; Benedek et al., 2006). Despite this criticism, TTC is still a reliable, rapid, and inexpensive method for analyzing enzymatically dysfunctional cells, most of which will eventually degenerate (Lust et al., 2002).

Because of the caveats described above, it becomes important to assess and confirm infarct size by other methods in addition to TTC, especially at the commonly used 24-h time point. Others have demonstrated that TTC and cresyl violet (CV) staining show a high degree of correlation in infarct area and volume at 24 h, indicating that both methods are suitable for producing accurate

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measurements of cerebral infarcts (Kudret et al., 2004). However, conventional histological techniques such as Nissl, hematoxylin and eosin (H&E), or CV stains also have limitations, as false positives occur due to processing artifacts or non-lethal alterations in cellular morphology (Schmued et al., 1997) and assessment is timely and labor intensive. These stains are also not specific for neuronal degeneration, as all cell types stain with these dyes. Additional relatively subtle morphological differences exist between normal and degenerating neurons making assessment more prone to bias.

Fluoro-Jade B is an anionic dye that specifically stains the soma and neurites of degenerating neurons by binding to a currently unknown basic substance in the neuron, most likely a poly-amine. It has the advantage of being as reliable and technically simple as a conventional Nissl stain, while being as specific for degenerating neurons as the "gold-standard" suppressed silver stain. It has a higher affinity for degenerating tissue components than Fluoro-Jade, reducing non-specific staining (Schmued and Hopkins, 2000). Recently Fluoro-Jade B has been used to identify neuronal degeneration secondary to ischemia (Schmued and Hopkins, 2000; Duckworth et al., 2005). Neuronal nuclear antigen (NeuN), a widely used marker for mature neurons, is expressed in nucleus and cell body of most neurons and not in glial cells, oligodendrocytes, astrocytes, or microglial cells (Wolf et al., 1996). Immunoreactivity for NeuN has been reported to decrease dramatically following CNS injury (e.g. MCAO and traumatic brain injury) (Igarashi et al., 2001; Davoli et al., 2002; Sugawara et al., 2002). However the loss of NeuN immunoreactivity may reflect injury-induced antigenicity rather than irreversible neuronal injury in ischemic models (Unal-Cevik et al., 2004).

In this study we used TTC, Fluoro-Jade B, and NeuN staining to examine the chronology of infarct development following MCAO. Our objectives were to determine if Fluoro-Jade B was superior to TTC staining, to delineate the time course of infarct progression, and to establish the anatomic boundaries of core and penumbra in mice at several time points after transient MCAO in mice.

2. Materials and methods

2.1. Animals

Male C57BL/6 mice (Charles River Laboratories) weighing 20–25 g at the time of surgery were used for all experiments. The mice were group-housed and maintained on a 12:12-h light/dark cycle, with ad libitum access to water and rodent chow. All procedures with animals were in accordance with the NIH guidelines for the care and use of animals in research and under protocols approved by the Animal Care and Use Committee of the University of Connecticut.

2.2. Ischemic model

Cerebral ischemia was induced by 90 min of MCAO under isoflurane anesthesia as previously described (McCullough et al., 2003). Briefly, rectal muscle temperature were monitored with a MONOTHERM system and maintained at approximately 37 °C during surgery and ischemia with an automated temperature control feedback system. A midline ventral neck incision was made, and unilateral MCAO was performed by inserting a silicone rubber-coated monofilament (Doccol Corp., CA) into the right internal carotid artery 6 mm from the internal carotid/pterygopalatine artery bifurcation via an external carotid artery stump. Sham animals underwent the same procedure but the suture was not advanced into the middle cerebral artery. Laser Doppler flow (Moor Instruments Ltd., England) was measured through the skull at the right temporal fossa (Sampei et al., 2000). Only the mice whose cerebral blood flow (CBF) showed a drop of over 85% of baseline just after MCAO were included which corresponds to dense ischemia as measured by quantitative blood flow methods (McCullough et al., 2005). Intra-ischemic neurological deficit was confirmed and scored as follows: 0, no deficit; 1, forelimb weakness and torso turning to the ipsilateral side when held by tail; 2, circling to affected side; 3, unable to bear weight on affected side; and 4, no spontaneous locomotor activity or barrel rolling. Reperfusion was documented with LDF.

2.3. TTC staining and measurement of brain infarct volume

Mice were euthanized at different time points of reperfusion. The brains were chilled at −80 °C for 4 min to slightly harden the tissue. Five, 2-mm coronal sections were made from the olfactory bulb to the cerebellum and then stained with 1.5% TTC (Sigma, St. Louise, MO). The stained brain sections were captured with a digital camera (MicroPublisher 5.0 RTV, QIMAGING). The infarct area of each brain was measured in a blinded manner, using an image analysis software, Sigmascan Pro 5. The infarct volume was calculated by Swanson's method (Swanson et al., 1990) to correct for edema. The total volumes of both contralateral and ipsilateral hemisphere, and the volumes of the striatum, cortex in both hemispheres were measured and the infarct percentage was calculated as % contralateral structure to avoid mis-measurement secondary to edema.

2.4. Fluoro-Jade B staining

Mice were anesthetized with pentobarbital and intracardially perfused with phosphate buffered saline (PBS) for 1 min followed by 4% paraformaldehyde in PBS for 30 min. Following perfusion, brains were dissected and post-fixed in 4% paraformaldehyde in PBS for 4 h. After post-fixation, brains were transferred to 20% sucrose (w/v) in 0.1 M phosphate buffer (PB) until equilibrated. The tissue was then frozen in Tissue-Tek OCT mounting medium and 30-μm coronal sections were cut and placed in 0.1 M PB. Sections were subsequently spread on microscope slides and allowed to air dry. Air dried sections were mounted on microscope slides and placed in 70% ethanol and ultrapure water for 3 min followed by 3 washes in ultrapure water for 1 min each rinse. Sections were oxidized by soaking in a solution of 0.06% $KMnO_4$ for 15 min then washed 3 times in ultrapure water 1 min each. Sections were subsequently stained in 0.001% Fluoro-Jade B (Chemicon International, CA) in 0.1% acetic acid for 20 min. Slides were subsequently washed 3 times in ultrapure water for 1 min each and dried overnight at room temperature. Dried slides were cleared in xylene and coverslips were mounted using Permount (Fisher Scientific). Digital images were collected on a Zeiss (Thornwood, NY) Axiovert 200M fitted with an apotome for optimal sectioning.

For the cortex and striatum, six 10× fields/animal and three 20× fields/animal were collected/animal ($n = 3$ per time point) respectively. Fluoro-Jade B positive cells were subsequently counted from each field using MacBiophotonics ImageJ software (NIH). For each animal, the total number of cells was averaged across fields of view for cortex, striatum, or total (cortex + striatum). These averages (avg. # cells/field of view) were used for statistical analysis.

2.5. NeuN staining

Brains were prepared and sectioned as described previously (see Fluoro-Jade B staining). Sections were subsequently mounted onto gelatin-coated coverslips and allowed to air dry. Air dried sections were blocked and permeabilized in 0.1 M PB with 0.3% TX-100 (sigma) and 10% goat serum (PBTGS) for 1 h. Following permeabilization, primary antibody (mouse monoclonal anti-NeuN 1:200; Chemicon International, Temecula, CA) was applied overnight at