Experimental Model of Brainstem Stroke in Rabbits via Endovascular Occlusion of the Basilar Artery

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Background: Basilar artery thrombosis remains a significant clinical problem, and no reproducible animal model has been established to study the stroke within the vertebrobasilar distribution. We report a study designed to pilot test a novel model of brainstem stroke in rabbits, created by selective endovascular occlusion of the basilar artery. Methods: Basilar artery occlusion was induced in 8 New Zealand white rabbits by injection of the autologous clot through the microcatheter positioned within the distal vertebral artery. Animals were divided into subgroups (I and II) based on the length of produced ischemia (3 and 6 hours, respectively). Magnetic resonance (MR) imaging of the brain and MR angiography of the intracranial vessels were performed before the procedure, and at 3 hours after induced ischemia for groups I and II, with continued imaging up to 6 hours for group II, with diffusion-weighted images acquired approximately every 30 minutes. Animals were killed at the end of the 3-hour (group I) or 6-hour (group II) ischemia time. Results: Brainstem stroke was successfully induced in all animals, with pathological changes documented in all cases. The earliest changes of ischemia on MR diffusion-weighted images were identified at only 4.5 hours of basilar artery occlusion. Conclusion: These results suggest that a reproducible model of brainstem stroke can be induced in rabbits using selective endovascular occlusion of the basilar artery. The availability of such a model, integrated with state-of-the-art imaging techniques, holds promise for preclinical investigations of emergent therapeutic approaches in stroke. Key Words: Basilar artery thrombosis—brainstem stroke—microtubule-associated protein-2 staining—axonal mitochondria.

Although basilar artery thrombosis remains a significant clinical problem, its prevalence is not known. Basilar artery occlusion has been reported in 2 of 1000 autopsy cases.1 It is estimated that 20% of all ischemic strokes occur in the vertebrobasilar system.2 Left untreated, basilar artery thrombosis has been associated with an 80% to 90% mortality. Even with successful recanalization of the basilar artery, the mortality remains at approximately 50%.3,4 Among survivors, up to 65% have poor neurologic outcomes.

Research on methods to treat basilar artery thrombosis has been hampered by the absence of validated animal models of brainstem stroke. No reproducible animal model has been established for the study of stroke within the vertebrobasilar distribution. We report here on a study designed to develop a model of brainstem stroke in rabbits, created by selective endovascular occlusion of the basilar artery.
Methods

After institutional animal care and use committee approval, feasibility studies were performed initially on two rabbits (data not shown) as to optimize magnetic resonance (MR) parameters for brain imaging, study vascular anatomy of the neck and head, and determine the most appropriate choice of embolic material (coils vs clot) for basilar artery occlusion.

After completion of this preliminary work, selective basilar artery occlusion was created in 8 New Zealand white rabbits (4 male, 4 female; 3.5-4.5 kg). Anesthesia was induced through isoflurane inhalation. After oral intubation, anesthesia was maintained with 1.5% isoflurane in air (oxygen mixture to provide fraction of inspired oxygen of 30%). All animals remained under general anesthesia with continuous monitoring of blood pressure, pulse oxymetry, capnography (end-tidal carbon-dioxide concentration), and core temperature.

After occlusion of the basilar artery, an increase in arterial blood pressure was noticed within a few minutes, with a mean increase of about 20% of the baseline. The increase declined over the next 2 hours without significant change in the heart rate. Animals were mechanically ventilated to keep end-tidal carbon-dioxide concentration at 30 to 35 mm Hg. No episodes of hemodynamic instability were observed with this model.

Arterial blood gas and blood glucose were monitored every 2 hours.

Procedure

A unilateral groin dissection was performed, the common femoral artery was punctured with a 4F micropuncture set, and a 4F sheath was placed. A right or left vertebral artery was selected with a 4F Bernstein angio- graphic catheter (Angiodynamics, Queensbury, NY). A microcatheter (Prowler-10, Boston Scientific, Natick, MA) was placed coaxially over a Mirage microwire (ev3, Irvine, CA) and manipulated into the distal vertebral artery using digital subtraction angiography, the roadmap technique, and fluoroscopic guidance (Fig 1, A). Animals were divided into subgroups (I and II, 4 animals in each group) based on the length of produced ischemia (3 and 6 hours, respectively). Occlusion of the basilar artery was obtained by injection of thrombus. Thrombus was prepared by mixing 2 mL of autologous blood with bovine thrombin (200 IU/mL) in a blood:thrombin ratio of 9:1. The volume of clot required to occlude the basilar artery was determined by calculating the cross-sectional area/length of the artery. That volume was doubled to allow for serum separation during extracorporeal clot formation. In a single case (in group II) the microcatheter was initially manipulated through the basilar artery and right posterior communicating artery into the distal left internal carotid artery, and thrombus was injected into the origin of the right middle cerebral artery (MCA), with occlusion of MCA flow documented by angiography (Fig 1, B). The microcatheter then was withdrawn back into distal left vertebral artery, and thrombus was injected to occlude the basilar artery, as in all other cases (Fig 1, C).

MR imaging (MRI) of the brain and MR angiography (MRA) of the intracranial vessels were performed at 3 hours after induced ischemia for groups I and II, with continued imaging up to 6 hours for group II. While still under general anesthesia, animals underwent craniotomy and were administered a lethal injection of barbiturates before brain extraction at the end of the 3-hour (group I) or 6-hour (group II) ischemia times.

Extracted brain specimens were sectioned into 1-mm slices (using Zivic matrix, Pittsburgh, PA) and maintained on ice. Infarcted areas in brain sections were identified by 2,3,5-triphenyltetrazolium chloride (TTC) staining.

Figure 1. Angiogram of vertebrobasilar territory through microcatheter placed into distal left vertebral artery (basilar artery [arrow]) (A). Occlusion of right MCA. Angiogram obtained through microcatheter positioned within distal basilar artery (left MCA [arrow]) (B). Occlusion of basilar artery after injection of autologous clot (C).
**MRI Protocol**

T1, T2, inversion recovery preparation, diffusion-weighted (DW), and fluid-attenuated inversion recovery MRI of the brain were obtained on a 3-T magnet. Fig 2 is an example of baseline imaging of the brain as part of the imaging protocol. Fractional anisotropy and relative anisotropy maps were created from the diffusion tensor imaging scans. Fiber tracts were generated from these maps. A time-of-flight technique was used to generate MRA images of the intracranial vessels before and after basilar artery occlusion (Fig 3).

**Pathology Protocol**

Preselected areas on extracted brain slices (similar to the planned MRI) were stained with TTC to identify areas of ischemia. TTC reduces to red formazan by functioning mitochondrial enzymes (specifically succinate dehydrogenase). Sections were incubated in 1.5% TTC in phosphate-buffered saline (PBS) for 30 minutes. These sections were then removed and placed in 4% formaldehyde. After 24 hours, fixed slices were sectioned at 5 μm and preserved in cryoprotectant solution for electron microscopy.

**Electron Microscopy**

Areas of the stroke that were fixed with 4% formaldehyde solution were also fixed in 2% glutaraldehyde in 0.1 mol/L cacodylate buffer with a pH of 7.4, washed well, and fixed for 30 minutes at 0°C in 1% osmium tetroxide in cacodylate buffer. After thorough washing, tissue was embedded in epoxy resin for thin sectioning. Sections were stained with uranium and lead, and evaluated for morphologic changes.

**Hematoxylin-eosin staining protocol**

Axial 5-μm tissue sections of brainstem were placed in PBS solution, stained with hematoxylin for 5 minutes, and washed in tap water. Slides were placed in 1% acid alcohol for a few seconds and, after a second wash in tap water, were placed in eosin for 5 minutes. After a final wash in tap water, slides were dehydrated and mounted on immunomount.

**Immunohistochemistry: microtubule-associated protein-2 staining**

Tissue was dehydrated through ascending alcohols, cleared in xylenes and embedded in paraffin. Five micron
sections were cut through the cortex and brainstem. Paraffin sections were placed in xylene, and then carried through descending alcohol series to water, rinsed in phosphate buffered saline (PBS, 0.05M, pH 7.4 containing .9% sodium chloride). The sections were rinsed in PBS, and placed into primary antibody in potassium PBS. Sections were kept for 1 h at room temperature and then for 48 h at 4°C. Slides were incubated in a moist chamber to prevent the tissue and antibody from evaporating during the incubation period. Tissue was rinsed multiple times. The primary antibody was monoclonal microtubule-associated protein (MAP-2A and 2B; Chemicon/Millipore, Billerica, MA) at 1:500. After rinsing, a drop of secondary antibody was placed on the sections and slides were kept for 1 hr at room temperature. The anti-mouse used was biotin-anti-mouse IgG (heavy and light chains -Vector Laboratories) at 1:600. After rinsing, an elite ABC kit from Vector laboratories

Figure 4. Gross pathology of extracted brain demonstrates dark clot (arrow) within basilar artery and pale ischemic brainstem compared with pink, nonthrombosed vessels along normal-appearing nonischemic brain convexity (A). TTC staining demonstrates ischemia (pale) within brainstem and cerebellum (B). Dark pink cerebral hemispheres as result of TTC reduced to red formazan by functioning mitochondrial enzymes within nonischemic brain parenchyma.

Figure 5. DW image demonstrates increased signal caused by restricted diffusion resulting from early changes of ischemia (at 4.5 hours) within brainstem (A). TTC staining: axial section of rabbit brainstem obtained after 6 hours of basilar artery occlusion. Section shows corresponding areas of brainstem stroke (white) (B).
was used for antigen detection. The slides were placed into A/B solution (4.5 μl each of solution A and solution B from Vector’s Vectastain® Elite ABC Kit per ml of PBS) for 1 hr at room temperature, rinsing them 3 times in PBS, and then 5 times in 0.175M sodium acetate solution. This is followed by placing the slides in chromogen solution consisting of 20 mg 3, 3’ diaminobenzidine tetrahydrochloride (Fluka brand, from Sigma catalog #3270 in 2.50 g Nickel (II) Sulfate hexahydrate (Sigma, catalog #N4882) per 100 ml sodium acetate with 83 μl of 3% peroxide added just prior to placement of the slides in the solution. Staining times are generally 15-20 minutes. After staining, the slides were rinsed in sodium acetate, followed by PBS, counterstained in a Nissl stain (neutral red), dehydrated through ascending alcohol series, cleared in xylenes and coverslipped with Permount. Sections were analyzed and photographed on a Nikon Eclipse 800 microscope fitted with a Retiga EX digital camera and captured using iVision software (Biovision Technologies, Exton, PA).

**Results**

Basilar artery occlusion was achieved in all subjects, as documented on catheter angiography. The artery remained occluded throughout the experiments based on MRA and gross pathology at the time of brain extraction (Fig 4, A). Pathologic changes of acute stroke within the pons were identified in all animals. Less consistent results were obtained at the level of cerebellum, medulla, and midbrain, probably secondary to the anatomic variability in blood flow from the posterior inferior cerebellar artery and the posterior cerebral artery. Areas of ischemia were identified by TTC staining (Fig 4, B) and loss of MAP-2

**Figure 6.** Electron microscopy at level of periphery of brainstem stroke area, showing mild-to-moderate changes in axonal mitochondria (single arrow) (A). Electron microscopy image of core of stroke with evidence of significant damage to axonal mitochondria (arrowhead) (B). Myelin sheath (double arrows) is more affected in core of stroke (A and B).

**Figure 7.** MAP-2 staining of cerebral cortex. Intact side shows strong MAP-2 immunoreactivity (black) within neurons (arrow) (A). On ischemic side, no MAP-2 immunoreactivity is preserved. Moreover, neurons appear shrunken, and no Nissl bodies are preserved in cytoplasm (arrowhead) (B).
staining as early as 3 hours after occlusion of the basilar artery and allowed preliminary work on quantification of the brainstem stroke volume. Immunohistochemical analysis showed significantly diminished MAP-2 staining at 6 hours of brain ischemia. MR DW imaging (3-T magnet) detected ischemia only after 4.5 hours of basilar artery occlusion and correlated well with areas of ischemia identified by TTC staining (Fig 5). Electron microscopy confirmed ischemia based on morphologic changes within the axonal mitochondria (Fig 6). When stroke was induced in both the basilar cerebral artery and MCA, both brainstem and cerebral cortex showed ischemic changes after 6 hours of ischemia (Figs 7 and 8). The brainstem neuronal cells, however, maintained better morphology (preserved Nissl bodies) and less loss of MAP-2 staining (Fig 8). Correlation was seen between areas of infarction detected by TTC staining and areas with diminished MAP-2 staining.

Discussion

A better understanding of cortical stroke has evolved from clinical data, imaging characteristics, experience with recanalization, and use of animal models. Guidelines for treatment have been based on imaging findings, and time windows have been established for different treatment options.7,8

During the last decade, information about cortical ischemia has been significantly enhanced by animal studies. The well-established and widely accepted rat stroke model is based on endovascular occlusion of the unilateral internal carotid artery and MCA origin by placing a filament through the carotid artery, dissected at the neck level.7 In general, endovascular occlusion is a preferred method for producing stroke when compared with the surgical approach involving craniotomy and placement of a clip or suture to occlude the vessel. The surgical approach produces additional trauma to the brain, causes intracranial pressure changes, and increases the risk of damage to the brain tissue and perforators.8

Stroke within the vertebrobasilar distribution behaves differently from cortical stroke. In human beings, outcomes in both treated and untreated vertebrobasilar stroke are significantly worse than those associated with cortical stroke. Patients with basilar occlusion respond to reperfusion in unpredictable ways, and neither the treatment window nor the optimal methods of recanalization has been defined.9,10

Established animal models have not contributed to knowledge about brainstem ischemia or potentially clinically translatable approaches to stroke treatment within the vertebrobasilar distribution. Two models to produce basilar occlusion in dogs, one via a skull-base surgical approach and vascular clip placement on the basilar artery and the other via thrombus injection through a catheter placed into the proximal vertebral arteries, have been proposed.11,12 Both models produced variable and unpredictable ranges of basilar artery occlusion, collateral flow, and volume of ischemia. Other models used coil occlusion of the vertebral arteries or a transthoracic surgical approach to the vertebral artery and direct injection of the clot into the vertebral artery allowing variable collateral flow as a result of unpredictable clot position.13,14

No in vivo MRI or pathologic changes/correlations of acute ischemia in vertebrobasilar distribution have been reported in an animal model. Rabbits are probably the lowest species in which reliable endovascular occlusion of the basilar artery, based on the size and anatomy of the vessels, is technically feasible.

The proposed model showed a reproducible complete occlusion of the basilar artery and infarction in the brainstem in all cases. The quantification of the brainstem stroke volume in our model is feasible based on TTC and MAP-2 staining. Less consistent results of stroke volume are expected within the cerebellum, because of anatomic variability of origin in the area supplied by posterior inferior cerebellar artery. Our model provided

Figure 8. MAP-2 staining of brainstem. Sections are counterstained for Nissl substance using neutral red. Staining at level of periphery of brainstem stroke, showing relatively preserved MAP-2 immunoreactivity (A). Within core of stroke MAP-2 staining is decreased, but neurons still show morphologic characteristics of healthy neurons (i.e., Nissl bodies are still evident). Cells are of relatively normal size (B).
selective intravascular occlusion of the basilar artery. Control catheter angiography and MRA confirmed the exact position of the thrombus. DW imaging detected changes at only 4.5 hours of ischemia—a finding that differs from commonly accepted and much earlier time frames for changes assessed by DW MRI in cortical stroke.

Evaluation of mitochondria from the stroke area demonstrated varying levels of severity of morphologic changes; as far as we are aware this is the first time such changes have been reported in brainstem stroke. Mitochondrial morphologic changes have been reported only in cortical stroke.\(^{15}\)

Immunohistochemical staining showed loss of MAP-2 staining both in the cerebral cortex and brainstem at 6 hours of ischemia, but the neurons of the cortex had more profound morphologic ischemic changes than brainstem neurons (Figs 7, B, and 8, B). Although these results were demonstrated only in a single case, this observation may indicate differential vulnerability, with brainstem neurons being more resistant to ischemia. This, in turn may be one of the explanations for positive outcome sometimes observed in patients with brainstem stroke treated late, outside of the traditional optimal time frame.\(^2\)

**Conclusion**

These results suggest that a viable and reproducible model of brainstem stroke can be induced in rabbits using selective endovascular occlusion of the basilar artery. The availability of such a model, integrated with state-of-the-art imaging techniques, holds promise for preclinical investigations of emergent therapeutic approaches in stroke.

**References**