New Pediatric Model of Ischemic Stroke in Infant Piglets by Photothrombosis

Acute Changes in Cerebral Blood Flow, Microvasculature, and Early Histopathology

John W. Kuluz, MD; Ricardo Prado, MD; Dansha He, MD; Weizhao Zhao, PhD; W. Dalton Dietrich, PhD; Brant Watson, PhD

Background and Purpose—The etiology and pathophysiology of acute ischemic stroke in children differ greatly from those in adults. The purpose of this study was to establish a new pediatric model of ischemic stroke in infant piglets for use in future studies of the response of the developing brain to focal ischemic injury.

Methods—Ischemic stroke was produced in male infant piglets (2 to 4 weeks old) by photothrombotic occlusion of the middle cerebral artery. Regional cerebral blood flow was measured with radiolabeled microspheres up to 4 hours after occlusion. Early histopathology, including caspase-3 immunohistochemistry for apoptosis, was examined 4 hours after ischemia. The nature of the thrombus and its interaction with vascular endothelium were assessed by electron microscopy.

Results—Severe ischemia (0 to 15 mL/100 g per min) occurred rapidly in 1.4±0.2 g of tissue at 15 minutes and increased to 2.4±0.7 g at 4 hours. Similarly, moderate ischemia (16 to 30 mL/100 g per min) was measured in 1.2±0.3 g of tissue at 15 minutes and increased to 2.0±0.6 g at 4 hours. These regional cerebral blood flow values represent ischemic levels of blood flow in 20% to 25% of the volume of the ischemic hemisphere at 4 hours after ischemia. Ischemic infarction occurred in both gray and white matter, and cerebral microvessels in the ischemic hemisphere contained large numbers of inflammatory leukocytes. Caspase-3–positive cells were few in number and were found in the periphery of the infarct; cell death appeared to occur primarily by necrosis rather than apoptosis at 4 hours. Electron microscopy revealed a pure platelet thrombus firmly attached to the vascular endothelium, which in some areas appeared to be detached from the basement membrane.

Conclusions—Ischemic stroke can be produced in infant piglets by middle cerebral artery photothrombosis. The stroke involved both gray and white matter and exhibited a robust inflammatory component. The mean infarct volume determined histopathologically amounted to 9.6±2.4% of the affected (ipsilateral) hemisphere, which was correlated well with the mass equivalent of tissue (12.0±3.5%), in which severe declines in regional cerebral blood flow were observed at 4 hours. (Stroke. 2007;38:1932-1937.)

Key Words: focal ischemia ■ neuroinflammation ■ apoptosis ■ photothrombosis ■ middle cerebral artery

Ischemic stroke is a serious yet poorly studied problem in infants and children with a variety of acute and chronic illnesses.1–2 Although the incidence in this age group of 3 to 6 cases per 100 000 per year is low compared with that in adults, the magnitude of the condition is amplified by the potential loss in long-term earning potential and quality-of-life years in the young child with stroke.3–5 Only 1 randomized trial of pediatric stroke treatment has been published describing a prevention strategy for children at risk of stroke due to sickle cell disease.6 Clinicians faced with managing a child with stroke have little solid evidence to support current treatment interventions.2–7 Clearly, there is an urgent need for clinical and basic science research in ischemic stroke in children.

The National Institute of Neurological Disorders and Stroke held an international workshop in 2001 to convene experts in the field of perinatal and childhood stroke8 who recognized that 1 of the deficiencies in pediatric stroke research was the “limited number of animal models for focal ischemic stroke” in the young. Studies of stroke in immature-animal models have shown that there clearly are age-related differences in the mechanisms of focal ischemic injury in the brain.9–11 These mechanistic differences suggest that treatment strategies to reduce ischemic injury in the immature...
brain need to be different compared with those for the mature brain. Although several rodent models of neonatal and pediatric stroke have been described,12–18 all have the disadvantage of not permitting repeated measurements of systemic physiologic variables and regional cerebral blood flow (rCBF) because of the small size of the animal subjects. The purpose of this study was to establish a new large-animal model of ischemic stroke in infant piglets by characterizing early rCBF changes and histopathology after photothrombotic occlusion of the middle cerebral artery (MCA).

Materials and Methods

General Surgical Preparation

Care and handling of the animals was approved by the University of Miami animal care and use committee. Eighteen piglets (5 to 7 kg, 2 to 4 weeks old) were used in this study. Piglets were anesthetized initially with pentobarbital 40 mg/kg IP followed by a bolus of fentanyl 50 μg/kg IV, fentanyl 5 μg/kg per hour IV, and pancuronium 0.2 mg/kg per hour IV. Additional doses of fentanyl 5 to 10 mg/kg IV were given as needed to maintain adequate anesthesia. Piglets were mechanically ventilated via tracheostomy to achieve normal arterial blood gases. A thermistor-tipped catheter was placed in the femoral vein and advanced into the right atrium for measurement of central venous pressure and core temperature and for drug and fluid administration. A femoral arterial catheter was placed for measurement of mean arterial blood pressure and arterial blood gases. For injection of radioactive microspheres, a catheter was placed in the contralateral femoral artery and advanced into the left ventricle with the use of waveform analysis. An auxiliary arterial catheter was placed to serve as the reference organ during microsphere injections. Finally, a catheter was placed in the superior sagittal sinus for measurement of cerebral venous oxygen content. Arterial pH and blood gases were analyzed (ABL-330, Radiometer, Copenhagen, Denmark) and were corrected for core temperature (pH-stat), which was kept at 38.0±0.5°C with the use of a warming blanket. Piglets received 0.9% saline 5 mL/kg per hour IV continuously and additional saline boluses of 10 mg/kg IV to keep central venous pressure at ≥4 mm Hg. Dextrose was given when the glucose concentration in whole blood decreased to <60 mg/dL. Sodium bicarbonate 1 to 2 mEq/kg IV was given when base deficit was >5 mmol/L and was unresponsive to saline fluid boluses. Cefazolin 30 mg/kg IV was also given at the beginning of each experiment. At the end of each experiment, piglets were killed with an overdose of KCl.

MCA Occlusion by Photothrombosis

The scalp was shaved, cleansed with Betadine, and reflected downward to expose the orbit. After enucleation of the eye, the orbital plate was removed with bone rongeurs, and a 1.5×2.0-cm craniotomy was performed to expose the left inferolateral MCA region. A U-shaped incision was made in the dura and reflected inferiorly. Two to three main and 1 to 3 smaller arteries were found supplying the MCA territory, usually arising from the circle of Willis, with considerable anatomic variability (Figure 1). For the purposes of this study, occlusion of a combination of the large and small arteries in the MCA region is referred to as MCA occlusion; only rarely could a single artery be found to supply the MCA territory after originating from the circle of Willis. Arterial occlusion was achieved by photothrombosis,19 in which a stable thrombus consisting solely of aggregated platelets is formed in response to endothelial peroxidative damage induced by type II photochemistry (ie, mediated by singlet molecular oxygen). The photochemical reaction was facilitated by the interaction of the intravenously injected photosensitizing dye erythrosin B (20 mg/kg) and the focused beam of an argon laser operated at 514.5 nm and power of 200 to 250 mW (Innova 70–4, Coherent, Inc, Palo Alto, Calif). The beam was directed at an average intensity of 60 to 75 W/cm² onto selected arteries supplying the MCA region (Figure 1). The arteries ranged in diameter from 360 to 1150 μm and averaged 650±360 μm (mean±SD; n=29). Larger-diameter arteries in this distribution usually required irradiation of up to 3 minutes at each of 2 different sites (distal site first), whereas the smaller ones required less time and a single exposure. In preliminary studies in 3 piglets, no ischemia was observed after occlusion of only the proximal portion of the large arteries, presumably due to compensatory vasodilation of collateral vessels.

Measurement of rCBF

Regional CBF was measured at baseline and after 15 minutes, 1 hour, and 4 hours of MCA occlusion with the use of radiolabeled microspheres. Eight piglets with stroke were studied, and 4 sham-operated time controls underwent the same procedures except for photothrombotic occlusion of the MCA. The preparation and use of microspheres for rCBF measurements followed previously validated protocols.20,21 Five isotopes were used (141Ce, 114In, 103Ru, 95Nb, and 99Sc), the sequence of which was randomized for each experiment. In brief, ~1.5×10⁶ latex microspheres (15±0.5 μm in diameter, Perkin-Elmer, Wilmington, Del), were injected into the left ventricle while arterial blood from the axillary artery was withdrawn at a rate of 1.91 mL/min to serve as the reference organ. The withdrawn blood was replaced with 0.9% saline (1:3, vol:vol). This dose of microspheres was sufficient to result in the presence of >400 microspheres in each tissue sample at baseline. At the end of the study, the brain was fixed in 10% buffered formalin (pH 7.4) and later cut into 0.5-cm coronal sections. The right and left hemispheres were carefully dissected into 47 regions of interest according to a standard grid. Brain tissue samples weighed between 0.2 and 30 mL/min per 100 g per minute. Tissue sections with severe ischemia, ie, the “core,” and moderate ischemia, ie, the “penumbra” (rCBF ≤15 and 16 to 30 mL/min per 100 g, respectively) are presented as mean±SEM.

Histopathology, Immunohistochemistry, and Electron Microscopy

An additional 4 piglets underwent photothrombotic ischemic stroke for early histopathologic analysis. Four hours after stroke, piglets were euthanized with KCl. Two sham-operated piglets served as controls. Brains were perfused in situ with use of a large transcardiac catheter placed in the ascending aorta to deliver 2 L of saline...
TABLE 1. Mean Arterial Pressure (MAP), Arterial PaCO2, and Core Temperature at Baseline and at 15 Minutes, 1 Hour, and 4 Hours After Photothrombosis in Piglets With Spontaneous Reperfusion and Permanent MCA Occlusion

<table>
<thead>
<tr>
<th>Physiologic Variable</th>
<th>Baseline (Mean ± SEM)</th>
<th>15 Minutes (Mean ± SEM)</th>
<th>1 Hour (Mean ± SEM)</th>
<th>4 Hours (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, bpm</td>
<td>160 ± 12</td>
<td>160 ± 10</td>
<td>153 ± 12</td>
<td>144 ± 12</td>
</tr>
<tr>
<td>MAP, mm Hg</td>
<td>104 ± 7</td>
<td>102 ± 5</td>
<td>105 ± 6</td>
<td>106 ± 6</td>
</tr>
<tr>
<td>PacO2, mm Hg</td>
<td>36.2 ± 0.4</td>
<td>37.8 ± 0.9</td>
<td>37.5 ± 0.8</td>
<td>38.2 ± 0.4</td>
</tr>
<tr>
<td>Temperature, °C</td>
<td>38.2 ± 0.1</td>
<td>38.3 ± 0.2</td>
<td>38.1 ± 0.2</td>
<td>38.2 ± 0.2</td>
</tr>
<tr>
<td>Hemoglobin, mg/dL</td>
<td>8.7 ± 1.1</td>
<td>9.5 ± 1.0</td>
<td>9.5 ± 1.0</td>
<td>9.0 ± 1.1</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>90 ± 10.1</td>
<td>88 ± 9</td>
<td>85 ± 7</td>
<td>102 ± 16</td>
</tr>
<tr>
<td>Lactate, mmol/L</td>
<td>1.2 ± 0.4</td>
<td>1.2 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. PaO2 was >100 mm Hg in all animals throughout the study period.

Statistical Analysis
Statistical analysis was performed with statistical software (CRUNCH Software Corp, Oakland, Calif). Physiologic variables and rCBF were analyzed by ANOVA for repeated measures. Values are expressed as mean ± SEM, with significance set at *P*<0.05.

Results

Heart rate, mean arterial pressure, core body temperature, glucose, lactate, hemoglobin, and arterial blood gases were stable and were not different from baseline throughout the study period (Table 1). All piglets survived 4 hours until study termination. There were no changes in blood pressure or heart rate due to injection of erythrosin B. After occlusion of all “MCA” vessels, cessation of large-vessel blood flow was easily seen through the operating microscope, leading to collapse of venules and loss of arterial pulsations. There were no tissue samples with ischemic levels of rCBF in either sham animals or at baseline in experimental piglets. Severe rCBF reductions occurred in all animals undergoing photothrombotic MCA occlusion. The mass equivalent of tissue with severe ischemia increased from 1.4 ± 0.2 g at 15 minutes to 2.4 ± 0.7 g at 4-hour MCA occlusion and with moderate ischemia from 1.2 ± 0.3 g to 2.0 ± 0.6 g, respectively (Figure 2). These changes in rCBF were different from baseline (*P*<0.001) but were not significantly different from each other over time, although there was a trend toward a further reduction of rCBF at 4 hours after ischemia. In these piglets, the average mass equivalent of each cerebral hemisphere was ~20 g; therefore, the sum of these reductions in rCBF amounted to 20% to 25% of the hemisphere. Regional CBF did not change significantly after onset of stroke in the cerebellum and hippocampus in either the ischemic (left) or nonischemic (right) side; however, in the left frontal cortex, rCBF decreased to 25% of baseline at 4 hours yet remained well above the ischemic threshold (Table 2).

Histopathologic examination revealed a moderate-size infarct involving both gray and white matter 4 hours after permanent MCA occlusion (Figure 3). The volume of infarct in the 4 brains examined 4 hours after stroke was 9.6 ± 2.4% (mean ± SD; range, 7.1% to 12.3%) of the ipsilateral hemisphere, or 1080 ± 330 mm³. The variance of the volume of infarct was 25%. A striking finding was the large number of cerebral vessels that contained, and in many cases were completely filled with, inflammatory leukocytes (Figure 4). Intraluminal leukocytes were found in a small number of vessels in the contralateral hemisphere but were not present in sham-operated piglets. Caspase-3 immunohistochemistry revealed that only a small number of cells along the infarct border contained immunoreactive product (Figure 5). Electron microscopy revealed a pure platelet thrombus adherent to the MCA

TABLE 2. Regional CBF Before and After Left MCA Occlusion by Photothrombosis

<table>
<thead>
<tr>
<th>Brain Region</th>
<th>Baseline (Mean ± SEM)</th>
<th>15 Minutes (Mean ± SEM)</th>
<th>1 Hour (Mean ± SEM)</th>
<th>4 Hours (Mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal cortex</td>
<td>Left: 90 ± 12</td>
<td>63 ± 6*</td>
<td>64 ± 5*</td>
<td>68 ± 6*</td>
</tr>
<tr>
<td></td>
<td>Right: 95 ± 15</td>
<td>78 ± 6</td>
<td>83 ± 5</td>
<td>93 ± 9</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>Left: 62 ± 9</td>
<td>51 ± 6</td>
<td>50 ± 6</td>
<td>52 ± 7</td>
</tr>
<tr>
<td></td>
<td>Right: 72 ± 11</td>
<td>62 ± 7</td>
<td>63 ± 5</td>
<td>66 ± 6</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>Left: 101 ± 14</td>
<td>92 ± 8</td>
<td>90 ± 8</td>
<td>97 ± 9</td>
</tr>
<tr>
<td></td>
<td>Right: 105 ± 15</td>
<td>95 ± 9</td>
<td>92 ± 7</td>
<td>99 ± 9</td>
</tr>
</tbody>
</table>

Values are mean ± SEM and are in mL/100 g per min.

*P*<0.05 for within-group comparisons by ANOVA for repeated measures.
vascular endothelium; the endothelium in this case was partially detached from the basement membrane (Figure 6).

**Discussion**

Our results demonstrate the feasibility of producing ischemic stroke in infant piglets, with reductions of rCBF to the ischemic range in 20% to 25% of the cerebral hemisphere. The surgical preparation is stable physiologically during the early period after stroke onset. Early histopathology revealing consistent infarction of both gray and white matter, a large number of inflammatory leukocytes adherent to cerebral microvessels, platelet activation and occlusive thrombus formation, and evidence of apoptotic cell death pathway activation are all important features of this piglet model of ischemic stroke. Although these data reflect only very early changes in rCBF and histopathology, they suggest that the photothrombotic method of MCA occlusion in infant piglets produces a reliable and clinically relevant large-animal model of human pediatric stroke. The mean volume of infarct of 9.6±2.4% of the affected hemisphere is well correlated with the mass equivalent of tissue (12.0±3.5%) with severely reduced rCBF values at 4 hours. Because of the permanent nature of the vascular occlusions in this model and the rCBF reductions it produces, the volume of infarct would likely increase with longer survival times.

Our method of measuring rCBF with microspheres in many small sections of brain tissue was designed for repeated measures over time to determine blood flow status within and around the area of ischemia. Decreasing the size of these samples improves the resolution but reduces the reliability of the flow measurements owing to an inadequate number of microspheres in the samples (<400), particularly during ischemia. However, the fact that the mass equivalent of tissue with severely reduced rCBF was correlated closely with the volume of ischemic infarct suggests that the blood flow values were sufficiently reduced to result in severe ischemic injury, at least within the core of ischemia at this early time point after stroke onset.

One explanation for the relative lack of scientific data regarding the response of the developing brain to stroke is the paucity of animal models of pediatric stroke.8 Models that require systemic hypoxemia are not clinically relevant to the child with stroke, because hypoxemia is uncommon in children with either ischemic or hemorrhagic stroke.1,2,4 Renolleau et al22 recently reported that reproducible normoxic stroke could be achieved in 7-day-old rat pups by permanently clipping the MCA and occluding the ipsilateral common carotid artery for 1 hour. Other investigators have produced ischemic stroke in developing rats as young as 10 days of age with the intraluminal suture method,14–16 brain injection of endothelin,17 and photothrombosis.18 However, the small size of rat pups at this age precludes measurement of important physiologic variables such as blood pressure, blood gases, and other variables that could impact recovery from ischemic stroke. The brains of larger animals such as pigs, dogs, and cats contain more white matter than those of...
rodents. This is important because of the differences in regional blood flow, metabolism, and injury mechanisms in white versus gray matter. Because no large-animal model has yet been established that mimics normoxic-ischemic stroke in children, establishing this infant piglet model of photothrombotic stroke should meet the need for a pediatric animal model for investigations of basic mechanisms of the developing brain’s response to focal ischemia and for preclinical studies of therapeutic interventions.

The photothrombotic method of vascular occlusion has gained widespread acceptance as a valid, noninvasive method for producing vascular injury, thrombosis, and associated ischemia in central nervous system tissue. For induction of arterial occlusion, the method involves directing the beam of an appropriate laser onto the desired artery after intravenous injection of a suitable photosensitizing dye. After absorption of laser light, the photoactive dye undergoes internal conversion to its metastable triplet state. Its electronic energy is then efficiently transferred to oxygen to create excited (singlet-state) oxygen, which then initiates direct endothelial injury by means of lipid (and possibly protein) peroxidation. Within seconds, a pure platelet thrombus begins to form and eventually results in complete occlusion of the irradiated arterial segment. This method of occlusion has several advantages over the traditional clip or other mechanically occlusive methods. Because the vessels are not surgically manipulated, there is little chance of direct trauma to vessels or surrounding brain tissue. Recruitment and activation of platelets are hallmarks of human thromboembolic stroke, and these responses are intentional and much more pronounced with photothrombosis than with other methods of arterial occlusion. Blood-borne substances are released from the site of arterial photothrombosis and can lead to acute alterations in the blood-brain barrier and worsening of ischemic injury. For these reasons, the photothrombotic method of MCA occlusion in piglets used in this study has great promise as a new and more inclusive model of pediatric ischemic stroke and the complications arising from it.

Age-related differences in injury and repair mechanisms in the brain in response to ischemic brain injury have been reported, and apoptosis is 1 that appears to be more involved in ischemic brain injury in the young compared with the old. Hu et al reported that >90% of neurons were caspase-3–positive in brain infarcts from immature rats after hypoxia-ischemia, whereas only 5% of neurons were caspase-3–positive in adult brain infarcts. Our data show that apoptotic cell death is not heavily involved in the process of normoxic-ischemic infarction in the early period after stroke. At later time points, however, apoptosis may play a greater role, and this requires further investigation.

A cascade of inflammatory processes has been shown to participate in the pathophysiology of several types of acute and chronic brain injuries, including stroke. Bona et al found that interleukin-1β and tumor necrosis factor-α were the first inflammatory cytokines expressed after hypoxia-ischemia in immature rats, followed by α-chemokines, β-chemokines, and finally neutrophils within vessels and brain parenchyma. In another developmental study, after intracerebral administration of interleukin-1β, upregulation of chemokines and invasion of neutrophils were greater in young than in adult rats. Our laboratory has reported a robust accumulation of leukocytes, both neutrophils and mononuclear cells, within cerebral microvessels 4 hours after an 8-minute period of cardiac arrest, although no intraparen-
chymal transmigration of leukocytes was observed. These inflammatory cells appeared to be associated with endothelial membrane blebs and blood-brain barrier breakdown. In our initial analysis of the early histopathology 4 hours after photothrombotic occlusion of the MCA, we found that many microvessels were literally filled with inflammatory leukocytes, with no intravascular leukocytes in sham-operated controls. Clearly, inflammatory cascades participate in focal ischemic injury in the developing brain, and this piglet model of photothrombotic ischemic stroke contains a strong inflammatory component.

Conclusions

Ischemic stroke can be produced in infant piglets by photothrombosis of the MCA. The early blood flow reductions and histopathology indicate that a moderately sized infarct will be produced by this method, which affects both gray and white matter and involves a robust inflammatory component. In future studies, we will examine long-term functional outcome, histopathological outcome, and mechanisms of injury and repair.

Acknowledgments

The authors thank J.P. Brunschwig for help with the electron microscopy, Susan Kraydieh for histopathologic processing, and Morayam Barreto and Yenis Mijares for secretarial assistance.

Sources of Funding

This study was supported in part by National Institutes of Health grants NS05820-32 (to W.D.D.) and R21NS044360 (to J.W.K.).

Disclosures

None.

References

New Pediatric Model of Ischemic Stroke in Infant Piglets by Photothrombosis: Acute Changes in Cerebral Blood Flow, Microvasculature, and Early Histopathology
John W. Kuluz, Ricardo Prado, Dansha He, Weizhao Zhao, W. Dalton Dietrich and Brant Watson

Stroke. 2007;38:1932-1937; originally published online April 26, 2007;
doi: 10.1161/STROKEAHA.106.475244
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2007 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/38/6/1932

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/