Perinatal Asphyxia in a Nonhuman Primate Model

Elizabeth N. Jacobson Misbe^a Todd L. Richards^b Ronald J. McPherson^a
Thomas M. Burbacher^c Sandra E. Juul^a

Departments of ^aPediatrics, ^bRadiology and ^cEnvironmental and Occupational Health Sciences,
University of Washington, Seattle, Wash., USA

Key Words

Hypoxic-ischemic encephalopathy · Neuroprotection ·
Perinatal asphyxia

Abstract

Perinatal asphyxia is a leading cause of brain injury in neonates, occurring in 2–4 per 1,000 live births, and there are limited treatment options. Because of their similarity to humans, nonhuman primates are ideal for performing preclinical tests of safety and efficacy for neurotherapeutic interventions. We previously developed a primate model of acute perinatal asphyxia using 12–15 min of umbilical cord occlusion. Continuing this research, we have increased cord occlusion time from 15 to 18 min and extended neurodevelopmental follow-up to 9 months. The purpose of this report is to evaluate the increase in morbidity associated with 18 min of asphyxia by comparing indices obtained from colony controls, nonasphyxiated controls and asphyxiated animals. Pigtail macaques were delivered by hysterotomy after 0, 15 or 18 min of cord occlusion, then resuscitated. Over the ensuing 9 months, for each biochemical and physiologic parameters, behavioral and developmental evaluations, and structural and spectroscopic MRI were recorded. At birth, all asphyxiated animals required resuscitation with positive pressure ventilation and exhibited biochemical and clinical

characteristics diagnostic of hypoxic-ischemic encephalopathy, including metabolic acidosis and attenuated brain activity. Compared with controls, asphyxiated animals developed long-term physical and cognitive deficits. This preliminary report characterizes the acute and chronic consequences of perinatal asphyxia in a nonhuman primate model, and describes diagnostic imaging tools for quantifying correlates of neonatal brain injury as well as neurodevelopmental tests for evaluating early motor and cognitive outcomes.

Copyright © 2011 S. Karger AG, Basel

Introduction

Perinatal asphyxia is a common cause of neonatal mortality and long-term disability, and accounts for 23% of neonatal deaths globally [1]. The application of early, moderate hypothermia to treat perinatal asphyxia has decreased the combined outcome of death or major disability from approximately 60 to 45% when strict diagnostic criteria are applied [2–5]. The efficacy of hypothermia is proof of concept that developmental brain injury can be mitigated, and it is possible that further improvement can be achieved by combining hypothermia with other neuroprotective treatments. Given that therapies are first developed in small-animal models, it is im-

perative that preclinical trials testing safety and efficacy also be developed for larger-animal models to more closely simulate human conditions. The nonhuman primate model is unique because of the physiologic and anatomic similarities to humans, and because neurodevelopmental tests adapted from those for humans can be used [6]. Primate models of perinatal brain injury were developed before the advent of sophisticated biochemical, imaging and immunohistochemical methods [7]. We have recently described a nonhuman primate model of acute perinatal asphyxia induced by 12–15 min of prenatal umbilical cord occlusion with subsequent neurodevelopmental testing to 4 months of age (approx. 16 human months) [8]. In this report, we have revised the protocol by extending cord occlusion to 18 min, optimizing the extensive MRI and continuing neurodevelopmental follow-up testing for 9 months (approx. 36 human months). This experiment is part of a larger study comparing the combined effects of high-dose erythropoietin (EPO) and hypothermia on outcomes after perinatal asphyxia. In this report, we compare the acute and chronic consequences of perinatal asphyxia of increasing duration without therapeutic hypothermia or EPO with non-asphyxiated controls, and highlight preliminary data suggesting that resuscitation techniques may have affected the outcome of asphyxia.

Methods and Animals

The animal care and use committees at the University of Washington in accordance with US NIH guidelines approved all experimental protocols. Figure 1 summarizes the study protocol.

Delivery and Resuscitation

Macaca nemestrina (pigtailed macaques) were delivered by hysterotomy under maternal general anesthesia with sevoflurane 1–8 days prior to term (173 days). After incising the uterus, the umbilical cord was exteriorized while keeping amniotic fluid and the fetus inside. To simulate perinatal asphyxia, the cord was clamped for either 15 or 18 min (asphyxiated group). Control animals were also delivered by hysterotomy, but did not undergo cord occlusion (control group). A 2.5-Fr Vygon™ umbilical arterial catheter was inserted prior to delivery. Fetuses were delivered, weighed and stabilized by a team of neonatologists using standardized neonatal resuscitation principles. Resuscitations included endotracheal intubation, positive pressure ventilation, chest compressions and bolus epinephrine as indicated, and Apgar scores were assigned. Monitoring included a pulse oximeter, rectal thermometer and amplitude-integrated electroencephalogram (aEEG; BrainZ BRM3, Natus Medical Incorp., San Carlos, Calif., USA). A covered heating pad, radiant warmer and polyethylene sheet were used to provide thermal support during stabilization, then the animals were moved to a thermal-neutral incubator.

Treatment Groups

For the parent study testing neuroprotective interventions, the asphyxiated animals were treated with saline injections, or with the neuroprotective treatments hypothermia (33°C for 72 h), EPO (1,000–3,000 i.v. U/kg/day $\times$ 4) or combined hypothermia plus EPO. For this preliminary report, data from the neuroprotection groups were not included. To focus on the effects of perinatal asphyxia, data from the saline-treated asphyxiated animals ($n = 12$) were compared with data from nonasphyxiated controls ($n = 5$). These data were also compared with developmental data from concurrent colony animals ($n = 20$).

Animal Care

The study animals were maintained for a minimum of 3 days on parenteral fluids (6–8% dextrose with 2% amino acids at 150 ml/kg/day to start) adjusted to maintain euglycemia and hydration. Enteral feedings were started on postnatal day (PND) 4 when the abdominal examination was normal and stooling was established. Feeds progressed from 50% Polycose/water to full infant formula (Similac®), then solid food as per Infant Primate Research Laboratory protocol. Weight was followed daily, and standardized anthropometric measurements were done every 2 weeks, including crown-rump length (CRL) and head circumference (HC) [9]. The Infant Primate Research Laboratory (IPRL) care protocol includes psychological and environmental enrichments [6]. Animals with seizure activity were administered repeated doses of phenobarbital 5 mg/kg (dose range: 5–30 mg/kg) until clinical seizure activity stopped. At 9 months of age, animals were sedated with ketamine, and then euthanized with an overdose of intravenous sodium pentobarbital. Animals were euthanized early if they exhibited neurologic morbidity that prevented unassisted breathing, prolonged inability to self-feed, or pneumonia unresponsive to antibiotics.

Developmental Evaluations

Early milestones included age at self-feeding and temperature stability. Newborn reflexes, muscle tone, behavioral state (attention, irritability and consolability) and neurological responses (visual and auditory perception) were assessed 5 days per week for the first 20 days, using tests based on the Brazelton Neonatal Behavioral Assessment Scale [8]. Neonatal activity and diurnals were collected every 4 h daily to determine onset or delay of diurnal cyclicity [10, 11]. Trained physical therapists – blinded to group assignment – evaluated spasticity, motor reflexes, passive range of motion and functional movement at scheduled time points, and performed an overall assessment of motor dysfunction.

Early cognition was evaluated using an object permanence testing paradigm beginning at PND 14 [12–14]. All infants were screened for deficits in visual acuity using a forced-choice preferential looking test [9, 15] before visual recognition memory testing (novelty preference) based on the Fagan Test of Infant Intelligence [16] beginning around PND 21 [17]. Social and motor behavior in mixed-sex play groups were scored by a trained observer 3 days per week [18, 19]. Around PND 145, infants were tested on a standard series of learning and memory problems utilizing the Wisconsin General Test Apparatus (WGTA) [20].

Laboratory Analyses

Point-of-care arterial blood gas and lactate (iSTAT®; HESKA Corp., Loveland, Colo., USA) were measured at multiple, scheduled intervals. Glucose and electrolytes were followed for titration

of parenteral fluids. Blood urea nitrogen, creatinine and aspartate transaminase were measured to assess end-organ injury.

MRI Acquisition and Analyses

All surviving asphyxiated animals, the control animals and 2 colony animals underwent MRI. Animals were preanesthetized for MRI with intramuscular ketamine (5–10 mg/kg) and atropine (0.04 mg/kg), then maintained on sevoflurane (0.8–2.5%) and 100% oxygen. Pulse oximetry and single-channel ECG were monitored with an MRI-compatible device (Invivo Precess™). Temperature was maintained with warm packs. The total scan time was approximately 2 h.

Two series of MRI were acquired for each animal, the first at 1 or 3 days, and the second at 6 or 9 months of age. Sequences included magnetization-prepared rapid gradient echo (MP-RAGE) high-resolution T₁-weighted imaging and single-voxel proton spectroscopy (MRS) acquired on a Phillips Achieva 3.0-tesla magnet with an X-series Quasar Dual gradient system. Two 8-channel array head coils were custom-made to fit neonatal and juvenile macaques.

Structural Analysis

A 3-D, high-resolution, T₁-weighted MP-RAGE protocol was used with the following parameters: multishot turbo field echo (TFE) pulse sequence with an inversion prepulse (1,151 ms delay); repetition time (TR)/echo time (TE) = 14 s/7.1 ms; 130 axial slices; acquisition matrix 208 × 141 × 130; acquisition voxel size 0.48 × 0.53 × 1.0 mm; reconstructed voxel size 0.39 × 0.39 × 0.5 mm; slice oversample factor = 2; sense factor = 2 in the foot-head direction; TFE factor = 141; number of signaling averages = 1; TFE shots = 65, and acquisition time = 3 min 14 s.

Volumetric Analysis

Analyze 9.0 (Mayo Clinic Biomedical Imaging Resource, Rochester, Minn., USA; 2009) was used for volume rendering of T₁-weighted images. Structures were isolated in the axial orientation using voxel-based intensity thresholds with manual limits. Specific boundaries for total brain and cerebellum were defined. Total brain volume included the cerebral hemispheres, superior sagittal sinus, diencephalon, brainstem, cerebellum and ventricular cerebrospinal fluid (CSF). Extra-axial CSF, optic chiasm and pituitary stalk were excluded, and the brainstem was truncated one slice caudal to the foramen magnum. The cerebellum was outlined separately, with the peduncles and brainstem excluded. Individuals masked to treatment group and outcome performed outlining in duplicate. The correlation between the two observers was >0.99. Final images were rendered in 3-D and inspected for accuracy before structural volumes were computed.

MR Spectroscopy

Single-voxel MRS was acquired using a point-resolved spectroscopy pulse sequence centered on a 10 × 10 × 10 mm voxel on the right thalamus. The MP-RAGE sequence was reconstructed in real time to guide MRS voxel placement. Acquisition parameters included: TR = 2,000 ms; TE at 6 different echo times (32, 45, 65, 80, 100 and 150 ms); 2,048 complex free induction decay points, and 2,000 Hz spectral width. Absolute concentrations of short-echo (at TE = 32 ms) metabolites: *N*-acetyl aspartate (NAA), creatine (Cr), choline (Cho), myoinositol (Ins), glutamate (Glu) and glutamate + glutamine (Glx) were calculated using LCModel [21]. During pre-

processing, the residual water signal was subtracted by decomposition-fitting. Free induction decays were then zero- and first-order phase corrected and smoothed using a 1.1-Hz exponential dampening filter. A nonlinear regression estimated the metabolite concentrations and uncertainties. Absolute concentrations were obtained by scaling the in vivo spectrum to the unsuppressed water peak and are reported in units that approximate millimolar concentrations. Partial volume corrections were made by calculating the fraction of CSF for each voxel using the FANTASM MIPAV (Fuzzy and Noise Tolerant Adaptive Segmentation Method Medical Image Processing, Analysis and Visualization) algorithm (Johns Hopkins Psychiatric Neuroimaging, Baltimore, Md., USA).

TE Phase Lactate

Lactate was analyzed separately from the other brain metabolites in order to isolate it from macromolecules/proteins. The lactate concentration was calculated from a new procedure developed to rephase the different echo time spectrum to give a lactate signal that was phased to stand straight up.

J-Resolved Spectroscopy

For each subject, a 2-D J-resolved acquisition sequence (JRES) was acquired from a 2 × 2 × 2 cm voxel centered on the thalamus. Parameters used were: point-resolved spectroscopy single-voxel pulse sequence; TE steps = 48 (32–502 ms, 10-ms increments); TR = 2 s; spectral bandwidth = 2,000 Hz; complex time points = 2,048; voxel dimensions 20 × 20 × 20 mm; number of excitations = 12, and total scan duration = 19.2 min [22, 23]. Spectra were acquired with and without water suppression (TE = 32 ms) for processing with LCModel [21, 24]. The raw 2-D MRS spectra (2,048 t₁ points × 48 t₂ points) were processed offline. Custom software was used to perform a 2-D Fourier transform, then the resulting signals were combined with LCModel to estimate metabolite concentrations. With a software called GAVA (www.briansoher.com), simulated JRES data were used to find the exact position of the J-coupled metabolites γ -aminobutyric acid (GABA) and glutamine. Gaussian filter adjustments were made to maximize separation of each metabolite.

Statistics

Univariate or mixed-model (for repeated measures) ANOVA was performed as appropriate before post hoc comparisons were made using software (SPSS version 17; SPSS Inc., Chicago, Ill., USA). Levene's test evaluated homogeneity. Parametric comparisons are presented as means with standard error (SEM) and numbers of animals. Nonparametric comparisons used the Mann-Whitney U test, and data are presented as scatter plots with medians. All comparisons were two-tailed, with $\alpha < 0.05$. Post hoc tests included the t test (one comparison) or Dunnett's test (multiple comparisons). The χ^2 or Fisher's exact test was used to compare proportional data.

Results

Mortality and Early Morbidity

Twelve *M. nemestrina* neonates delivered by hysterotomy after cord occlusion did not undergo therapeutic hypothermia or receive EPO (n = 5 at 15 min; n = 7 at 18 min).

Table 1. Birth characteristics comparing colony and study animals

	Colony OT N/A (n = 20)	Control OT 0 min (n = 5)	Asphyxiated	
			OT 15 min (n = 5)	OT 18 min (n = 7)
Gestational age, days	174.2 ± 1.4	168.4 ± 1	168.2 ± 1.2	168.7 ± 0.9 ^a
Gestational age range, days	164–187	166–172	166–171	166–171
Birth weight, g	560 ± 13	537 ± 35	572 ± 28	554 ± 25
Birth CRL ¹ , mm	207.7 ± 1.5	207.4 ± 3.4	204.0 ± 2.7 (4)	203.5 ± 2.8 (6)
Birth HC ¹ , mm	211.9 ± 1.4	201.4 ± 1.7 ^b	210.6 ± 3.4 (4)	205.8 ± 3.1 (6)

Values denote means ± SEM (with n in parentheses) unless otherwise specified. ANOVA found effects of colony for gestational age ($F_{1,36} = 5.4$; $p = 0.026$) and HC ($F_{1,36} = 10.9$; $p = 0.002$) and results of Dunnett's post hoc comparisons are indicated as ^a $p < 0.05$ and ^b $p < 0.01$ compared with colony animals. OT = Occlusion time. ¹ Two initial anthropometric measurements were unavailable.

Table 2. Resuscitation characteristics for nonasphyxiated control and asphyxiated neonatal primates

	Control OT 0 min (n = 5)	Asphyxiated	
		OT 15 min (n = 5)	OT 18 min (n = 7)
Endotracheal intubation, n	2 (40%)	5 (100%)*	7 (100%)*
Chest compressions, n	0	4 (80%)*	7 (100%) [†]
Bolus epinephrine, n	0	4 (80%)*	7 (100%) [†]
Apgar score			
1 min	3.2 ± 1.0	0.8 ± 0.4*	0.4 ± 0.2 [‡]
5 min	5.2 ± 0.4	1.6 ± 0.2 [‡]	2.1 ± 0.5 [‡]
10 min	8.0 ± 0	2.8 ± 0.4 [‡]	2.9 ± 0.3 [‡]
20 min	8.0 ± 0	3.6 ± 0.5 [‡]	4.3 ± 0.3 [‡]
Blood gas ¹			
Initial pH	6.99 ± 0.07 (3)	6.82 ± 0.05*	6.77 ± 0.02 [‡]
Initial CO ₂ , mm Hg	71.7 ± 2.3 (3)	97.3 ± 17.1	112.2 ± 9.0
Peak base deficit, mM/l	6.7 ± 3.5	20.2 ± 1.9 [‡]	20.3 ± 0.9 [‡]
Peak lactate, mM/l	10.8 ± 2.6	13.8 ± 0.8	14.0 ± 0.6

Values denote means ± SEM (with n or percentages in parentheses) unless otherwise specified. Proportions were evaluated by Fisher's exact test. Repeated measures ANOVA found a time × occlusion time interaction for Apgar scores ($F_{3,6} = 3.1$; $p = 0.013$), and ANOVA found effects of occlusion time for pH ($F_{2,14} = 5.9$; $p = 0.016$), base deficit ($F_{2,16} = 7.8$; $p = 0.005$) and lactate ($F_{2,16} = 6.6$; $p = 0.01$). Fisher's test and Dunnett's post hoc tests are indicated as * $p < 0.05$, [†] $p < 0.01$ and [‡] $p < 0.001$ compared with controls. OT = Occlusion time. ¹ Two initial blood gas measurements were unavailable.

Death occurred 3 days after asphyxia in 2 animals due to neurorespiratory abnormalities. Table 1 compares the birth characteristics of colony (n = 20), control (n = 5) and these untreated, asphyxiated (15 vs. 18 min) animals. To date, there have been no sex differences on any measure, so data from males and females are combined. Compared with colony animals, control and asphyxiated (study) animals had a lower gestational age at birth, and the controls had smaller HC, but other measures did not differ.

Table 2 compares the resuscitation procedures, early laboratory results and Apgar scores for control versus asphyxiated animals. Though 2 of the 5 nonasphyxiated control animals required brief intubation (1 due to secondary apnea), none required chest compressions or epinephrine. In contrast, 11 of the 12 asphyxiated animals required chest compressions and received intravenous epinephrine for a heart rate of less than 60 bpm. As shown, all mean Apgar scores, initial pH values and peak

Fig. 1. Timeline illustrating the protocol for a nonhuman primate model of perinatal asphyxia. In utero, animals undergo umbilical cord occlusion (cord clamp) for 15–18 min, then are delivered by hysterotomy and resuscitated. Erythropoietin or vehicle and/or hypothermia treatments may be applied during the first week.

Data collected include early EEG, MRI and MR spectroscopy (MRS), as well as growth and neurodevelopmental assessments to 9 months of age. BrdU = Bromodeoxyuridine; PT Eval = physical therapist evaluation; IHC = immunohistochemistry.

Fig. 2. Base deficit following perinatal asphyxia in primates treated with bicarbonate administration or without. The metabolic acidosis due to umbilical cord occlusion normalized in 2–3 h whether or not bicarbonate was administered.

Fig. 3. Graph plotting 6 months of individual daily weights for colony, control, 15-min and 18-min asphyxia animals. **Inset** Weekly weight gain per caloric intake for the first 3 postnatal months. The growth of study animals was compromised despite adjustments to provide adequate calories.

base deficits were significantly worse in asphyxiated animals. All asphyxiated primates met standard diagnostic criteria for human perinatal asphyxia with low Apgar scores and need for positive pressure ventilation at 10 min, severe metabolic acidosis within the first 60 postnatal minutes, and depressed physical examination and aEEG [2–5]. Despite the severe metabolic acidosis present at birth, arterial pH normalized by 2–3 h regardless of whether sodium bicarbonate was administered (fig. 2).

Results of the initial neurologic examination of all asphyxiated animals were profoundly abnormal, with no spontaneous respirations or movement, no withdrawal from painful stimuli, and flaccidity. Early aEEG tracings were also abnormal for the first 3–6 postnatal hours, after which hypotonia evolved into myoclonic activity. None of the control animals had seizures, while 8 of the 12 asphyxiated animals exhibited clinical seizures at an average of 9.0 ± 1.7 h of age.

Fig. 4. Scatter plots with medians (line) plotting the developmental indices for groups of primates. Asphyxia delayed early thermal stability (**a**, **b**), self-feeding (**c**) and formula weaning (**d**) as well as the age at which animals were willing to separate from the sur-

rogate (**e**). There were no effects on toy exploration (**f**). ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$, nonparametric Mann-Whitney U comparisons compared with colony; * $p < 0.05$, † $p < 0.01$, compared with control.

Growth

Perinatal asphyxia compromised somatic growth. Figure 3 tracks the daily growth of control and asphyxiated animals superimposed upon the growth of colony animals. The inset in figure 3 tracks the weight gain per caloric intake of study animals for the first 3 postnatal months. Animals asphyxiated for 18 min weighed an average of 15% less at 3 months compared with controls ($p = 0.03$). Asphyxiated animals fed poorly and frequently required formula of increased caloric density (up to 24

kcal/oz) to achieve weight gain. The weight gain of the saline-treated, asphyxiated animals improved but did not normalize during the study. Delayed growth was also indicated by a significant 2–3% decrease in both CRL and HC at 1 and 6 months postmenstrual age (data not shown).

Developmental and Behavioral Testing

Neurodevelopmental indices were adversely affected by perinatal asphyxia. Figure 4 plots early developmental

Table 3. Nursery assessments and motor and cognitive indices for groups of primates

	Colony OT N/A	Control OT 0 min	Asphyxiated	
			OT 15 min	OT 18 min
Nursery assessments, days to criterion				
Muscle tone	4.3 ± 0.4 (20)	5.5 ± 0.2 (5)	7.6 ± 0.1 (4)*	9.1 ± 0.7 (6) [‡]
Response quality	3.9 ± 0.3 (20)	6.2 ± 0.6 (5) ^a	6.8 ± 0.8 (4) ^b	8.8 ± 1.1 (6) ^c
Early reflexes	6.1 ± 0.4 (20)	6.9 ± 0.5 (5)	7.7 ± 0.7 (4)	10.1 ± 1.3 (6)
Behavioral state	5.0 ± 0.6 (20)	6.9 ± 1.2 (5)	10.1 ± 2.7 (4)	9.7 ± 1.4 (5)
Object permanence, days to criterion				
Reach	30.3 ± 2.4 (20)	31.6 ± 4.2 (5)	37.3 ± 5.3 (4)	46.2 ± 3.2 (6)
Screen hide	69.3 ± 5.1 (20)	86.8 ± 5.8 (5)	81.3 ± 4.3 (4)	89.5 ± 10.1 (4)
Well hide	71.9 ± 6.2 (20)	88.0 ± 14.3 (5)	86.5 ± 14.9 (4)	94.3 ± 12.4 (4)
Motor skills, days to criterion				
Climb ramp	27.8 ± 3.3 (20)	24.6 ± 2 (5)	56.8 ± 12.4 (4)*	40.5 ± 4.8 (6)
Release grasp	40.5 ± 4.7 (20)	42.2 ± 8.3 (5)	76.5 ± 14.2 (4)	64.7 ± 10.9 (6)
RIFM (dexterity)	61.7 ± 5.3 (20)	100 ± 11.3 (4) ^a	79.3 ± 4.6 (4)	119 ± 29.3 (3) ^b
WGTA, sessions to criterion				
B/W discrimination	2.9 ± 0.3 (16)	2.8 ± 0.8 (4)	3.8 ± 0.5 (4)	4.6 ± 1.2 (5)
B/W reversal	4.6 ± 0.4 (16)	5.5 ± 0.6 (4)	5.5 ± 1.2 (4)	5.3 ± 0.9 (4)
Color discrimination	5.4 ± 1.2 (16)	12.0 ± 3.9 (4)	11.8 ± 3.7 (4)	7.4 ± 1.2 (5)
Hamilton Search Adaptation	4.0 ± 0.5 (16)	4.5 ± 1.2 (4)	5.3 ± 0.8 (4)	6.3 ± 1.3 (4)
Recognition memory, % net preference for novel image ¹				
Abstract	9.6 ± 2.6 (19) [‡]	11.2 ± 2.8 (5) [†]	10.29 ± 8.4 (4)	4.72 ± 8.5 (5)
Social	4.8 ± 2.1 (19)*	1.1 ± 1.3 (5)	4.7 ± 1.9 (4)*	0.1 ± 4.6 (5)
Overall	7.3 ± 1.6 (19) [‡]	6.0 ± 1.2 (5) [†]	7.7 ± 3.8 (4)	2.3 ± 2.8 (5)

Values denote means ± SEM with numbers in parentheses. OT = Occlusion time; RIFM = relatively independent finger movements; B/W = black/white.

¹ Fagan Test of Infant Intelligence: normal animals spend significantly more time focused on a novel image relative to chance. ^a $p < 0.05$, ^b $p < 0.01$, ^c $p < 0.001$, Dunnett's post hoc test compared with colony; * $p < 0.05$, [†] $p < 0.01$, [‡] $p < 0.001$, compared with control (or with chance for Fagan tests of memory).

Table 4. Physical therapy overall assessments

	Number	1 week	1 month	4 months	8 months ⁴
Colony	11/20 ¹	11 normal	11 normal	11 normal	10 normal
Control	5/5	5 normal	5 normal	5 normal	4 normal
Asphyxiated (15 min)	4/5 ²	3 normal; 1 severe	3 normal; 1 moderate	4 normal	3 normal; 1 mild
Asphyxiated (18 min)	6/7 ^{2, 3}	1 normal; 3 mild; 2 moderate	2 normal; 4 mild	2 normal; 3 mild	3 normal; 1 mild

¹ A subset of colony animals underwent standardized evaluations. ² 2 animals died prior to evaluation. ³ One 4-month evaluation not available. ⁴ 4 animals were not yet 8 months old at the time of data analysis.

milestones and exploratory behaviors. Asphyxia delayed the development of temperature stability, prolonging incubator and heating pad support (fig. 4a, b), and also delayed self-feeding and formula weaning (fig. 4c, d). In addition, asphyxia delayed the animals' separation from the

surrogate, but did not alter exploration of novel toys (fig. 4e, f). The data in table 3 compare the early nursery assessments and subsequent motor and cognitive indices. Asphyxia increased early muscle tone and impaired performance on resistance, grasping and clasping tasks. The

quality of responses to a battery of nursery assessments that included startle, auditory orientation, righting, and visual orientation and following was delayed in all pre-term study animals, both asphyxiated and nonasphyxiated, compared with the colony animals. In the animals asphyxiated for 18 min, there was no effect on early reflexes ($p = 0.06$) or behavioral state.

Table 3 also shows that motor and cognitive deficits were present in study animals. Most notably, although there were no significant effects of asphyxia on tests of object permanence or on the WGTA assessments involving visual discrimination, asphyxia impaired the primates' capacity to recognize objects that had previously been presented (novelty preference). Recognition memory deficits were greatest in animals exposed to 18 min of cord occlusion. When post hoc analyses were indicated to allow direct comparison between animals undergoing 15 and 18 min of cord occlusion, there were no significant differences in the results of developmental testing.

Table 4 presents the physical therapy overall assessment for groups of animals. As shown, all colony and control animals were normal. Although, initially, some asphyxiated animals exhibited indications of motor dysfunction, by 8 months of age, only 2 animals exhibited mild impairment, 1 each asphyxiated for 15 and 18 min. The most severely affected animal had been asphyxiated for 15 min and underwent physical therapy to assist with mobility and self-feeding.

MRI and MRS

Asphyxia did not significantly affect macroscopic brain growth, but may have influenced brain chemistry. Figure 5 plots the changes in total brain and cerebellar volumes for colony/control (combined) and asphyxiated animals. There were no differences in total brain volume. On average, brain size increased by 50% from baseline to 6 months, with another 4% increase from 6 to 9 months. Cerebellar volume between asphyxiated and control animals at 6 months of age was marginally, insignificantly different ($p = 0.055$).

Table 5 lists the mean brain metabolite concentrations detected on MRS in animals grouped by cord occlusion time at the different time points. Several metabolite concentrations increased with age (Cr, NAA, Glu1 and Glx) and several decreased (Cho, macromolecules, Ins and TE phase lactate). With the exception of Cho, there were no effects of asphyxia and no interactions between asphyxia and age detected for the other metabolites listed. There were also no effects of asphyxia on the ratios TE phase

Fig. 5. Scatter plots showing MRI measurements of total brain (a) and cerebellar volumes (b) in groups of primates. Mixed-model ANOVA found that total brain volumes increased over time ($F_{3,7} = 43.6$; $p < 0.001$), but there were no group differences. Cerebellar volumes increased over time ($F_{3,7} = 44.9$; $p < 0.001$), and there was a marginally nonsignificant effect of occlusion time ($F_{2,8} = 3.57$; $p = 0.055$), thus post hoc testing was not performed. For colony/control, 15-min and 18-min animals, respectively, the numbers of animals imaged were: 7, 2 and 4 at 1 day; 3, 1 and 3 at 3 days; 2, 3 and 0 at 180 days, and 5, 2 and 4 at 270 days.

lactate/NAA, NAA/Cho, Cho/Cr and NAA/Cr (ratios not shown). Cho was affected by asphyxia ($F_{2,22} = 3.9$; $p = 0.035$), but post hoc comparisons for specific occlusion time were not significant at each age. However, post hoc evaluation for effects of age indicated that Cho increased from day 1 to day 3 in the animals asphyxiated for 18 min. Because there were no significant effects of asphyxia (or asphyxia interactions) for other metabolites, testing of age effects stratified by occlusion time was not warranted.

Table 5. MRS concentrations at different ages of animals undergoing different umbilical cord occlusion times

	Occlusion time, min	1 day mM/kg	3 day mM/kg	6 months mM/kg	9 months mM/kg
Cho	0	1.56 ± 0.06 (7)	1.51 ± 0.05 (4)	1.24 ± 0.11 (2)	1.36 ± 0.06 (5)
	15	1.59 ± 0.04 (2)	1.86 ± 0.14 (3)	1.34 ± 0.05 (3)	1.57 ± 0.08 (2)
	18	1.41 ± 0.03 (4)	1.82 ± 0.12 (3)*		1.43 ± 0.09 (4)
Cr	0	5.45 ± 0.20 (7)	5.41 ± 0.17 (4)	6.31 ± 0.54 (2)	6.85 ± 0.11 (5)
	15	5.32 ± 0.05 (2)	5.65 ± 0.20 (3)	7.18 ± 0.34 (3)	6.97 ± 0.15 (2)
	18	5.35 ± 0.14 (4)	5.67 ± 0.31 (3)		6.80 ± 0.33 (4)
NAA	0	4.29 ± 0.14 (7)	4.26 ± 0.18 (4)	5.75 ± 0.05 (2)	6.03 ± 0.22 (5)
	15	4.34 ± 0.22 (2)	3.87 ± 0.25 (3)	5.79 ± 0.29 (3)	5.90 ± 0.08 (2)
	18	3.91 ± 0.12 (4)	4.10 ± 0.02 (3)		6.34 ± 0.15 (4)
Macromolecules	0	10.24 ± 0.77 (7)	9.87 ± 0.72 (4)	10.77 ± 0.86 (2)	9.25 ± 0.92 (5)
	15	9.06 ± 1.40 (2)	13.42 ± 3.05 (3)	10.16 ± 1.16 (3)	8.35 ± 0.34 (2)
	18	10.06 ± 0.82 (4)	12.08 ± 0.69 (3)		6.72 ± 1.05 (4)
Ins	0	4.49 ± 0.24 (7)	4.69 ± 0.14 (4)	4.21 ± 0.78 (2)	4.12 ± 0.10 (5)
	15	5.03 ± 0.99 (2)	5.39 ± 0.97 (3)	4.34 ± 0.31 (3)	4.70 ± 0.30 (2)
	18	4.44 ± 0.19 (4)	5.24 ± 0.08 (3)		3.50 ± 0.17 (4)
TE phase lactate	0	0.46 ± 0.03 (7)	0.33 ± 0.10 (4)	0.36 ± 0.05 (2)	0.37 ± 0.06 (5)
	15	0.49 ± 0.08 (2)	0.77 ± 0.57 (3)	0.26 ± 0.03 (3)	0.12 ± 0.01 (2)
	18	0.46 ± 0.06 (4)	0.60 ± 0.05 (3)		0.33 ± 0.08 (4)
Glu1	0	3.02 ± 0.13 (7)	3.25 ± 0.07 (4)	4.37 ± 0.20 (2)	3.72 ± 0.22 (5)
	15	2.85 ± 0.60 (2)	4.51 ± 0.86 (2)	3.83 ± 0.24 (3)	3.60 ± 0.52 (2)
	18	3.06 ± 0.11 (4)	2.84 ± 0.28 (3)		3.59 ± 0.42 (4)
Glx	0	8.22 ± 0.51 (7)	7.36 ± 0.76 (4)	10.26 ± 1.05 (2)	9.79 ± 0.59 (5)
	15	8.00 ± 0.22 (2)	7.82 ± 1.28 (3)	10.37 ± 0.45 (3)	9.18 ± 0.39 (2)
	18	8.35 ± 0.37 (4)	8.13 ± 0.50 (3)		9.94 ± 0.58 (4)
JRES glutamine	0	0.74 ± 0.10 (7)	0.63 ± 0.09 (2)	0.59 ± 0.02 (2)	0.93 ± 0.15 (5)
	15			0.46 ± 0.06 (2)	0.66 ± 0.03 (2)
	18	0.61 ± 0.17 (4)	0.75 ± 0.22 (3)		0.95 ± 0.18 (4)
JRES GABA	0	0.56 ± 0.10 (7)	0.56 ± 0.02 (2)	0.67 ± 0.11 (2)	0.78 ± 0.11 (5)
	15			0.40 ± 0.17 (2)	0.46 ± 0.19 (2)
	18	0.57 ± 0.17 (4)	0.59 ± 0.12 (3)		0.75 ± 0.14 (4)

Values denote means ± SEM with numbers in parentheses. Mixed-model ANOVA were performed, and there were significant effects of age and occlusion time for Cho, and effects of age for Cr, NAA, Glu1, macromolecules, Ins, Glx and TE lactate. The F statistics are omitted for brevity here. A significant post hoc comparison of Cho is shown as * $p < 0.05$ compared with day 1.

Parent Study post hoc Analyses

To consider post hoc possible influences of birth and resuscitation factors on outcome after asphyxia, we assessed such details between the least affected, the nonsurviving and the most severely affected animals in the parent study. The 4 least affected animals (2 from 15-min and 2 from 18-min group) were born 2–6 days early and were intubated within 6 min of birth after receiving bag-

mask ventilation. Three received epinephrine by 2 min of age (1 did not require chest compressions or epinephrine) and none received bicarbonate. Of the 4 animals that did not survive a week, 3 were born more than 1 week early, 2 had 5 intubation attempts each, and 2 were given bicarbonate. The 3 most severely affected animals were born 2–4 days early, underwent multiple intubation attempts, and 1 was given bicarbonate. These animals had very

poor early weight gain and poor brain growth (1 had the smallest total brain volume at 6 months).

As the parent study progressed, the developmental course of the animals asphyxiated for 15 min improved. During the first year of the study, 2 animals died in the first postnatal week; in the second year, 2 were not independently mobile in the nursery, and after 2 years of the study, those asphyxiated for 15 min all survived and none exhibited any persistent, major, qualitative neurologic issues except 1 with minor contractures.

Discussion

The major findings of this preliminary report are that 15 and 18 min of umbilical cord occlusion can produce severe, acute birth asphyxia with subsequent delays in growth and development. These data confirm that *M. nemestrina* may be used to model human perinatal asphyxia. Using serial MRI, we demonstrated brain growth and developmental changes over time for a variety of brain metabolites. Significant asphyxia-induced differences in subacute MRS metabolites (e.g. increased lactate, decreased NAA) and brain growth were not detected, likely due to increased within-group variability and small group numbers. It is also possible that acute changes in brain lactate may have resolved before the initial imaging at 24 h of age. These data raise concern that the use of MRS in human clinical trials needs to be validated; however, there are promising reports that used MRS after perinatal asphyxia [25–28]. Utilizing multifaceted developmental testing, we identified physiologic, physical and functional differences resulting from perinatal asphyxia.

We previously reported data from a pilot study of 8 primates that underwent 12–15 min of umbilical cord occlusion to produce perinatal asphyxia with developmental testing to 4 months [8]. In the pilot study, 15 min of asphyxia produced severe spasticity, contractures and motor impairment. In the current study, 15 min of asphyxia produced less motor impairment despite comparable average initial pH, base deficit and Apgar scores between the two studies. Differences in the severity of injury between the pilot and the current study may reflect differences in the degree of prematurity (current study: 5 ± 2 early; pilot study: 7 ± 1 days early), or in the timing of umbilical catheter placement (current study: before delivery; pilot study: after delivery). Also, for the current study, we discontinued the use of sodium bicarbonate to treat acidosis as part of resuscitation. Moreover, resusci-

tation team experience has improved over time, as has the recognition and treatment of aspiration pneumonia in the primate nursery. We speculate that a more effective team can more immediately and efficiently resuscitate the animals, which likely reduces the complications of acute asphyxia. In addition to prematurity [29], duration of asphyxia, resuscitation measures and nursery care, individual genetics and in utero conditions may also influence susceptibility to brain injury from asphyxia. In the current study, cord occlusion was extended from 15 to 18 min to increase the severity of injury, but despite that change, there was no increase in mortality and little change in developmental, physical and behavioral outcomes.

Animal models of human disease permit the discovery of pathophysiologic mechanisms and therapeutic opportunities. Though small-animal models using mice [30], rats [31] and rabbits [32] to examine neonatal brain injury have provided informative data, larger-animal models that use piglets [33, 34], sheep [35] and nonhuman primates [7, 36, 37] provide additional essential information needed to begin clinical trials. Large-animal models are preferable because these animals may have complex neurological features that are more comparable to humans, such as similar gray/white matter proportioning and substantial cortical folding. Sophisticated MR techniques may now be used to measure the chemical and structural composition of the neonatal brain, and these techniques should be thoroughly validated using sensitive animal models in order to improve the prognosis of developmental abnormalities following perinatal asphyxia [38]. To that end, this nonhuman primate model of perinatal asphyxia enables sophisticated behavioral and methodological testing and biological discovery that cannot be performed on human infants.

The objective of the parent study is to test the safety and efficacy of EPO plus hypothermia for the treatment of perinatal hypoxic-ischemic encephalopathy. Ultimately, results will be evaluated longitudinally and cross-sectionally between treatment groups to assess the effects of the combined treatments. The use of the nonhuman primate has provided the opportunity to evaluate biochemical, behavioral and structural features of this disease process and its treatment. Postmortem histological evaluation of the brain using immunochemistry and electron microscopy will allow for correlation with in vivo MR findings. Assessment of systemic organs will provide additional safety information. This animal model is an important tool to permit preclinical evaluation of the safety and efficacy of new therapies. The de-

developmental testing is particularly useful for examining staged treatments. We expect that the optimal treatment for perinatal hypoxic-ischemic encephalopathy will require multiple interventions timed for different phases of injury [39].

Acknowledgments

We would like to give special thanks to: Jeff Stevenson of the University of Washington Magnetic Resonance Research Laboratory; Cecil E. Hayes, PhD, of the University of Washington Radio-frequency Laboratory; the Washington National Primate Re-

search Center and Infant Primate Research Laboratory, including Pat Delio, Noelle McKain and Steve Ellis; Brittany Baker, Sarah Hays, Marianne Bricker and Kelly Ledbetter from the Juul laboratory, and Elizabeth Aylward, PhD, of the Seattle Children's Research Institute. We also thank Olympic Medical for the donation of a cooling unit and the BrainZ BRM3 brain monitor.

This study was supported by NIH grants HD52820 and HD02274 through the National Institute of Child Health and Human Development and ARRA (American Recovery and Reinvestment Act of 2009) to S.E.J., and also by University of Washington Institute of Translational Health Sciences National Center for Research Resources grants RR025014, RR025015 and RR025016, and the Washington Regional Primate Research Center base operating grant RR000166.

References

- Black RE, Cousens S, Johnson HL, Lawn JE, Rudan I, Bassani DG, Jha P, Campbell H, Walker CF, Cibulskis R, Eisele T, Liu L, Mathers C: Global, regional, and national causes of child mortality in 2008: a systematic analysis. *Lancet* 2010;375:1969–1987.
- Gluckman PD, Wyatt JS, Azzopardi D, Ballard R, Edwards AD, Ferriero DM, Polin RA, Robertson CM, Thoresen M, Whitelaw A, Gunn AJ: Selective head cooling with mild systemic hypothermia after neonatal encephalopathy: multicentre randomised trial. *Lancet* 2005;365:663–670.
- Shankaran S, Laptook AR, Ehrenkranz RA, Tyson JE, McDonald SA, Donovan EF, Fanaroff AA, Poole WK, Wright LL, Higgins RD, Finer NN, Carlo WA, Duara S, Oh W, Cotten CM, Stevenson DK, Stoll BJ, Lemons JA, Guillet R, Jobe AH: Whole-body hypothermia for neonates with hypoxic-ischemic encephalopathy. *N Engl J Med* 2005;353:1574–1584.
- Perlman JM: Summary proceedings from the neurology group on hypoxic-ischemic encephalopathy. *Pediatrics* 2006;117:S28–S33.
- Azzopardi DV, Strohm B, Edwards AD, Dyet L, Halliday HL, Juszczak E, Kapellou O, Levene M, Marlow N, Porter E, Thoresen M, Whitelaw A, Brocklehurst P: Moderate hypothermia to treat perinatal asphyxial encephalopathy. *N Engl J Med* 2009;361:1349–1358.
- Burbacher TM, Grant KS: Methods for studying nonhuman primates in neurobehavioral toxicology and teratology. *Neurotoxicol Teratol* 2000;22:475–486.
- Myers RE: Four patterns of perinatal brain damage and their conditions of occurrence in primates. *Adv Neurol* 1975;10:223–234.
- Juul SE, Aylward E, Richards T, McPherson RJ, Kuratani J, Burbacher TM: Prenatal cord clamping in newborn *Macaca nemestrina*: a model of perinatal asphyxia. *Dev Neurosci* 2007;29:311–320.
- Dobson V, McDonald MA, Kohl P, Stern N, Samek M, Preston K: Visual acuity screening of infants and young children with the acuity card procedure. *J Am Optom Assoc* 1986;57:284–289.
- Sackett GP, Fahrenbruch CE, Ruppenthal GC (eds): Development of Basic Physiological Parameters and Sleep-Wakefulness Patterns in Normal and At-Risk Neonatal Pig-tail Macaques (*Macaca nemestrina*). New York, Plenum, 1979, pp 125–142.
- Sackett GP, Ruppenthal GC: Nursery Rearing of Nonhuman Primates in the 21st Century. New York, Kluwer Academic, 2006.
- Mishkin M, Appenzeller T: The anatomy of memory. *Sci Am* 1987;256:80–89.
- Diamond A, Goldman-Rakic P: Comparison of human infants and rhesus monkeys on Piaget's AB task: evidence for dependence on dorsolateral prefrontal cortex. *Exp Brain Res* 1989;74:24–40.
- Burbacher TM, Grant KS, Mottet NK: Retarded object permanence development in methylmercury exposed *Macaca fascicularis* infants. *Dev Psychol* 1986;22:771–776.
- Teller DY: The development of visual acuity in human and monkey infants. *Trends Neurosci* 1981;4:21–24.
- Fagan JF, Singer LT (eds): Infant Recognition Memory as a Measure of Intelligence. New York, Ablex, 1983.
- Gunderson VM, Sackett GP: Development of pattern recognition in infant pigtailed macaques (*Macaca nemestrina*). *Dev Psychol* 1984;20:418–426.
- Sackett GP, Stephenson E, Ruppenthal GC: Digital data acquisition systems for observing behavior in laboratory and field settings. *Behav Res Methods Instrum* 1973;5:344–348.
- Castell R, Sackett G: Motor behaviors of neonatal rhesus monkeys: measurement techniques and early development. *Dev Psychobiol* 1973;6:191–202.
- Harlow HF, Harlow MK, Rueping RR, Mason WA: Performance of infant rhesus monkeys on discrimination learning, delayed response, and discrimination learning set. *J Comp Physiol Psychol* 1960;53:113–121.
- Provencher SW: Estimation of metabolite concentrations from localized in vivo proton nmr spectra. *Magn Reson Med* 1993;30:672–679.
- Jensen JE, Frederick BD, Wang L, Brown J, Renshaw PF: Two-dimensional, J-resolved spectroscopic imaging of GABA at 4 Tesla in the human brain. *Magn Reson Med* 2005;54:783–788.
- Jensen JE, Frederick Bde B, Renshaw PF: Grey and white matter GABA level differences in the human brain using two-dimensional, J-resolved spectroscopic imaging. *NMR Biomed* 2005;18:570–576.
- Provencher SW: Automatic quantitation of localized in vivo ¹H spectra with LCModel. *NMR Biomed* 2001;14:260–264.
- L'Abbe C, de Vries LS, van der Grond J, Groenendaal F: Early diffusion-weighted MRI and ¹H-magnetic resonance spectroscopy in asphyxiated full-term neonates. *Biol Neonate* 2005;88:306–312.
- Miller SP, Vigneron DB, Henry RG, Bohland MA, Ceppi-Cozzio C, Hoffman C, Newton N, Partridge JC, Ferriero DM, Barkovich AJ: Serial quantitative diffusion tensor MRI of the premature brain: development in newborns with and without injury. *J Magn Reson Imaging* 2002;16:621–632.
- Cappellini M, Rapisardi G, Cioni ML, Fonda C: Acute hypoxic encephalopathy in the full-term newborn: correlation between magnetic resonance spectroscopy and neurological evaluation at short and long term. *Radiol Med* 2002;104:332–340.
- Mañeru C, Junqué C, Bargalló N, Olondo M, Botet F, Tallada M, Guardia J, Mercader JM: ¹H-MR spectroscopy is sensitive to subtle effects of perinatal asphyxia. *Neurology* 2001;57:1115–1118.

- 29 MacDonald HM, Mulligan JC, Allen AC, Taylor PM: Neonatal asphyxia. I. Relationship of obstetric and neonatal complications to neonatal mortality in 38,405 consecutive deliveries. *J Pediatr* 1980;96:898–902.
- 30 Fagel DM, Ganat Y, Silbereis J, Ebbitt T, Stewart W, Zhang H, Ment LR, Vaccarino FM: Cortical neurogenesis enhanced by chronic perinatal hypoxia. *Exp Neurol* 2006;199:77–91.
- 31 Kellert BA, McPherson RJ, Juul SE: A comparison of high-dose recombinant erythropoietin treatment regimens in brain-injured neonatal rats. *Pediatr Res* 2007;61:451–455.
- 32 Derrick M, Luo NL, Bregman JC, Jilling T, Ji X, Fisher K, Gladson CL, Beardsley DJ, Murdoch G, Back SA, Tan S: Preterm fetal hypoxia-ischemia causes hypertonia and motor deficits in the neonatal rabbit: a model for human cerebral palsy? *J Neurosci* 2004;24:24–34.
- 33 Thoresen M, Hallström A, Whitelaw A, Puka-Sundvall M, Løberg EM, Satas S, Ungerstedt U, Steen PA, Hagberg H: Lactate and pyruvate changes in the cerebral gray and white matter during posthypoxic seizures in newborn pigs. *Pediatr Res* 1998;44:746–754.
- 34 Thoresen M, Satas S, Løberg EM, Whitelaw A, Acolet D, Lindgren C, Penrice J, Robertson N, Haug E, Steen PA: Twenty-four hours of mild hypothermia in unsedated newborn pigs starting after a severe global hypoxic-ischemic insult is not neuroprotective. *Pediatr Res* 2001;50:405–411.
- 35 Gunn AJ, Bennet L, Gunning MI, Gluckman PD, Gunn TR: Cerebral hypothermia is not neuroprotective when started after postischemic seizures in fetal sheep. *Pediatr Res* 1999;46:274–280.
- 36 Myers RE: Threshold values of oxygen deficiency leading to cardiovascular and brain pathological changes in term monkey fetuses. *Adv Exp Med Biol* 1973;37:1047–1053.
- 37 Myers RE: Fetal asphyxia due to umbilical cord compression: metabolic and brain pathologic consequences. *Biol Neonate* 1975;26:21–43.
- 38 Lodygensky GA, Inder TE, Neil JJ: Application of magnetic resonance imaging in animal models of perinatal hypoxic-ischemic cerebral injury. *Int J Dev Neurosci* 2008;26:13–25.
- 39 Gonzalez FF, Ferriero DM: Neuroprotection in the newborn infant. *Clin Perinatol* 2009;36:859–880, vii.