Concurrent Erythropoietin and Hypothermia Treatment Improve Outcomes in a Term Nonhuman Primate Model of Perinatal Asphyxia

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Key Words

Hypoxic-ischemic encephalopathy · Spectroscopy · Diffusion tensor imaging · Cerebellum · Pharmacokinetics · Choline

Abstract

Background: Up to 65% of untreated infants suffering from moderate to severe hypoxic-ischemic encephalopathy (HIE) are at risk of death or major disability. Therapeutic hypothermia (HT) reduces this risk to approximately 50% (number needed to treat: 7–9). Erythropoietin (Epo) is a neuroprotective treatment that is promising as an adjunctive therapy to decrease HIE-induced injury because Epo decreases apoptosis, inflammation, and oxidative injury and promotes glial cell survival and angiogenesis. We hypothesized that HT and concurrent Epo will be safe and effective, improve survival, and reduce moderate-severe cerebral palsy (CP) in a term nonhuman primate model of perinatal asphyxia. **Methodology:** Thirty-five *Macaca nemestrina* were delivered after 15–18 min of umbilical cord occlusion (UCO) and randomized to saline (n = 14), HT only (n = 9), or HT+Epo (n = 12). There were 12 unasphyxiated controls. Epo (3,500 U/kg × 1 dose followed by 3 doses of 2,500 U/kg, or Epo 1,000 U/kg/day × 4 doses) was given on days 1, 2, 3, and 7. Timed blood samples

were collected to measure plasma Epo concentrations. Animals underwent MRI/MRS and diffusion tensor imaging (DTI) at <72 h of age and again at 9 months. A battery of weekly developmental assessments was performed. **Results:** UCO resulted in death or moderate-severe CP in 43% of saline-, 44% of HT-, and 0% of HT+Epo-treated animals. Compared to non-UCO control animals, UCO animals exhibit poor weight gain, behavioral impairment, poor cerebellar growth, and abnormal brain DTI. Compared to UCO saline, UCO HT+Epo improved motor and cognitive responses, cerebellar growth, and DTI measures and produced a death/disability relative risk reduction of 0.911 (95% CI –0.429 to 0.994), an absolute risk reduction of 0.395 (95% CI 0.072–0.635), and a number needed to treat of 2 (95% CI 2–14). The effects of HT+Epo on DTI included an improved mode of anisotropy, fractional anisotropy, relative anisotropy, and volume ratio as compared to UCO saline-treated infants. No adverse drug reactions were noted in animals receiving Epo, and there were no hematology, liver, or kidney laboratory effects. **Conclusions/Significance:** HT+Epo treatment improved outcomes in nonhuman primates exposed to UCO. Adjunctive use of Epo combined with HT may improve the outcomes of term human infants with HIE, and clinical trials are warranted.

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Introduction

Hypoxic-ischemic (HI) brain injury at birth remains a significant problem, affecting 1.3–1.7/1,000 term live-born infants in the USA and contributing to 23% of neonatal deaths globally [1, 2]. Untreated, moderate to severe HI encephalopathy (HIE) carries a 60–65% risk of either death or major neurodevelopmental disability, including mental retardation, cerebral palsy (CP), hydrocephalus, and seizures [3, 4]. Therapeutic hypothermia (HT) in term infants reduces this risk to approximately 50%, with a number needed to treat ranging from 7 to 9 [3]. The efficacy of HT provides proof of concept that HI brain injury can be mitigated; however, additional treatments are needed to further improve outcomes.

HIE evolves over more than a week [5–7]. Therapeutic HT targets early mechanisms of HI injury by reducing cerebral metabolism, excitotoxic neurotransmitter accumulation, ATP depletion, oxygen and nitrogen free radical release, and lipid peroxidation of cell membranes [8–10]. Ideally, supplementary HIE treatments will complement the mechanisms of HT neuroprotection [11, 12]. Erythropoietin (Epo) is a potentially promising supplementary therapy to augment brain repair and improve neurodevelopment outcomes. Small animal models of HIE have demonstrated that high-dose Epo treatment decreases neuronal apoptosis, oligodendrocyte injury, inflammation, oxidative injury, nitric oxide toxicity, and glutamate toxicity while increasing neurogenesis, oligodendrogenesis, glial cell proliferation, and angiogenesis [13, 14]. We hypothesize that Epo+HT will be a safe and effective combination therapy that will improve survival and prevent the development of moderate-severe CP after HIE in a term nonhuman primate model of perinatal asphyxia [15].

Methods

Animals

The Animal Care and Use Committees at the University of Washington in accordance with US National Institutes of Health (NIH) guidelines approved all experimental protocols. Fifty-six *Macaca nemestrina* (pigtailed macaques) were delivered for the purposes of this study. Forty-two animals were delivered after umbilical cord occlusion (UCO) for either 15 or 18 min. UCO subjects were assigned to 1 of 4 treatment groups: saline, Epo only, HT only, or HT+Epo. Nine animals of similar gestational age were delivered by cesarean section to serve as normal controls. The first 25 treatment assignments were simple randomization with the order of treatments determined by sorting a sequence of random numbers, and subsequent assignments followed adaptive randomization

such that the sex of the animal at birth determined the treatment assignment until the groups were balanced; this was done in an effort to minimize gender effects [16]. Longitudinal neurodevelopment, anthropometric data, and spectroscopy data were also collected from concurrent controls ($n = 5$).

Delivery and Resuscitation

The use of UCO to model perinatal asphyxia in *M. nemestrina* has previously been reported and is summarized in figure 1 [15, 17]. UCO was performed on animals in utero 1–8 days prior to term (173 days). The uterus was incised and the umbilical cord was externalized and clamped for 15 or 18 min (asphyxia group). While the clamp was in place, the uterus was supported with saline-soaked towels and an umbilical artery catheter was installed. After the UCO, the fetuses were delivered by hysterotomy. Control animals were also delivered by hysterotomy after intrauterine installation of an umbilical arterial catheter (2- to 3-min procedure). Fetuses were delivered and stabilized by a team of neonatologists using standardized neonatal resuscitation practice. Resuscitations included endotracheal intubation, positive pressure ventilation, chest compressions, and bolus epinephrine as indicated. Apgar scores were assigned at 1, 5, 10, and 20 min. Monitoring included a pulse oximeter, rectal thermometer, and amplitude-integrated EEG (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, and then the animals were moved to a thermal-neutral incubator.

Treatment Groups

UCO animals were treated with saline, Epo, HT, or Epo+HT. Epo treatment was either 3,500 U/kg $\times$ 1 dose i.v. followed by 3 i.v. doses of 2,500 U/kg given at 30 min, 24 h, 48 h, and 7 days or Epo 1,000 U/kg/day i.v. $\times$ 4 dosages at 30 min, 24 h, 48 h, and 7 days (Epogen®, epoetin alfa recombinant; Amgen) [15]. The initial Epo dosing was based on the 2,500 U/kg used in the phase I/II clinical trial [18] (with the addition of a 3,500-U/kg loading dose), but the dosing was lowered to 1,000 U/kg because the initial pharmacokinetics parameters were 25% higher than expected, possibly due to organ dysfunction or to HT. To produce HT, the animals were not actively warmed at delivery. Active cooling was begun after resuscitation of the infants and always occurred by the third hour of life. To maintain HT, a water blanket was applied to the animal's head and thermal support from the incubator was adjusted to achieve a rectal temperature of 33.5°C for 72 h (Olympic Medical Cool Care System; Olympic Medical, Seattle, Wash., USA). Rectal temperature was maintained by adjusting the incubator temperature. Rewarming was done slowly, raising the rectal temperature by 0.5°C/h.

Animal Care

Postresuscitation care was conducted as previously reported [15]. Briefly, arterial blood gas, lactate, and electrolytes (iSTAT®; HESKA Corp., Loveland, Colo., USA) were measured at multiple, scheduled intervals. The study animals were maintained for a minimum of 3 days on parenteral fluids adjusted to maintain euglycemia and hydration. Enteral feedings were started on postnatal day 4, after rewarming for infants who were treated with HT, when the abdominal exam was normal and stooling was established. Weight was followed daily and standardized anthropometric measurements were done every week, including crown-rump length and head circumference.

Fig. 1. Schedule of procedures and assessments for the nonhuman primate model of perinatal asphyxia. MRI = Magnetic resonance and diffusion tensor imaging and spectroscopy; PT = physical therapy. ^a The first MRI scan was performed at either 24 or 72 h of age.

Pharmacokinetic Analysis

Blood samples (0.1 ml/sample) were collected to measure plasma Epo concentrations at timed intervals: 0 (predose baseline), 3, 6, 12, 24, 48, and 72 h as previously described [18]. Data analysis was conducted using noncompartmental pharmacokinetic techniques. The trapezoidal rule was used to compute the area under the plasma concentration-versus-time curve (AUC) until the last measured value at 24 h. The AUC was extended to infinity by taking the quotient of the 24-hour concentration and the elimination rate constant. $C_{\max}$ is the maximum plasma concentration observed after the first dose.

The $t_{1/2}$ for the plasma Epo concentration-versus-time curve was computed by dividing 0.693 by the elimination rate constant. Clearance (Cl), volume of distribution [using the steady-state (V_{ss}) and area (V_{area}) methods], and mean residence time (MRT) were calculated using the following formulas: $Cl = D/AUC$, $V_{ss} = (D \times AUMC)/AUC^2$, $V_{area} = D/(k \times AUC)$, and $MRT = AUMC/AUC$, where D is the recombinant Epo dose and AUMC is the area under the first moment curve.

Developmental Evaluations

Developmental assessment was performed by the Infant Primate Research Laboratory at the University of Washington, as previously described [15]. Briefly, age at self-feeding and temperature stability, newborn reflexes, muscle tone, behavioral state, and neurological responses were assessed 5 days/week for the first 20 days using tests based on the Brazelton Neonatal Behavioral Assessment Scale. Neonatal activity was recorded every 4 h. Evaluators who were blinded to treatment assigned an overall assessment of motor dysfunction. The object permanence testing paradigm, screening for deficits in visual acuity, novelty preference, social and motor behavior in mixed-sex play groups, and a standard series of learning and memory problems utilizing the Wisconsin General Test Apparatus were administered by trained evaluators.

To determine the presence or absence of CP, animals were assessed by a physical therapist with a special interest in neonates. Sequential exams were done at 1 week, 1 month, and 8 months to document any evidence of motor abnormalities and contractures.

Assessment included evaluation of their ability to control active movement. Muscle tone at each joint was graded on the Ashford scale of 0 (normal) to 4 (affected parts rigid in flexion or extension) [19].

MRI Acquisition and Analyses

All surviving asphyxiated animals, control animals, and 5 colony animals underwent sedated MRI as previously described at 24 or 72 h and again at 9 months [15]. The total scan time was approximately 2 h and consisted of magnetization-prepared rapid gradient echo (MPRAGE) high-resolution T1-weighted imaging, diffusion tensor imaging (DTI), and single-voxel proton spectroscopy (MRS) acquired on a Philips 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. Details of the sequence acquisition were performed as previously reported [15].

Volumetric Analysis. Manual 3-D tracings of the whole brain, cerebellum, and caudate were created using the interactive semi-automated tools within RView software (<http://rview.colin-studholme.net/>), and the corresponding volumes were calculated [20]. Tracings were performed by 3 separate observers who were blind to the treatment. Specific boundaries for total brain, caudate, and cerebellum were defined. The total brain volume included the cerebral hemispheres, superior sagittal sinus, diencephalon, brainstem, and cerebellum. The ventricular and extra-axial cerebrospinal fluid, optic chiasm, and pituitary stalk were excluded, and the brainstem was truncated at 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 interrater reliability of brain segmentation was determined by Dice similarity coefficients. The Dice similarity coefficients between the 3 tracers ranged from 0.885 to 0.921 for caudate, 0.9255 to 0.9862 for cerebellum, and 0.9135 to 0.9738 for cortex. Final images were rendered in 3-D and inspected for accuracy before structural volumes were computed.

DTI. Voxelwise statistical analysis of the fractional anisotropy (FA) data was carried out using tract-based spatial statistics [21]

(TBSS), part of the FMRIB Software Library (FSL) [22]. First, FA images were created by fitting a tensor model to the raw diffusion data using FDT, and they were then brain-extracted using BET [23]. All subjects' FA data were then aligned into a common space using the nonlinear registration tool FNIRT [24, 25], which uses a b-spline representation of the registration warp field [26]. Representative control animal scans at 3 days and 9 months were used to align animals instead of the human normal space. Next, the mean FA image was created and thinned to create a mean FA skeleton, which represents the centers of all tracts common to the group. Each subject's aligned FA data were then projected onto this skeleton, and the resulting data were fed into voxelwise cross-subject statistics. Voxelwise cross-subject statistics on non-FA DTI data (first eigenvalue, second eigenvalue, third eigenvalue, relative anisotropy, mean diffusivity, mode of anisotropy, volume ratio, radial diffusivity, and raw T2 signal without diffusion weighting) were performed using the `tbss_non_FA` command. Threshold-free cluster enhancement with correction for multiple comparison results was used to determine significance.

MR Spectroscopy. Single-voxel MRS was acquired using the point-resolved spectroscopy (PRESS) pulse sequence centered on a $10 \times 10 \times 10$ -mm voxel on the right thalamus in order to calculate the absolute concentrations of N-acetyl aspartate, creatine, choline, myo-inositol, glycine, glutamate, and glutamine as previously described using LCModel [15, 27, 28].

TE Phase Lactate. Lactate was analyzed separately from the other brain metabolites in order to isolate it from macromolecules/proteins as previously described [15].

J-Resolved Spectroscopy. For each subject, a 2-D J-resolved acquisition sequence was acquired from a $2 \times 2 \times 2$ -cm voxel centered on the thalamus as previously described [15]. Custom software was used to perform a 2-D Fourier transform and then the resulting signals were combined with LCModel to estimate metabolite concentrations. With software called GAVA (www.briansoher.com), simulated JRES data were used to find the exact position of the J-coupled metabolites gamma-aminobutyric acid (GABA) and glutamine. Gaussian filter adjustments were made to maximize the separation of each metabolite.

Statistics

DTI was compared by voxelwise statistical analysis and can compare only 2 groups at once [21]. Levene's test evaluated homogeneity. Parametric comparisons are presented as means with standard error (SEM) and numbers of animals. All comparisons were two-tailed, with $\alpha \leq 0.05$. Two analyses were conducted. First, validation that the model produces injury was determined by comparing control animals to any animal that received UCO. Second, determination of the neuroprotection of treatments was performed with the exclusion of the control group. Post hoc testing of treatment effects was compared to UCO saline as the control group. Death or moderate-severe CP and epinephrine, sodium bicarbonate, and phenobarbital administration were evaluated by cross-tabs with the binary output as the row and asphyxia (yes or no) as one column (Pearson's χ^2) and treatment (saline, HT, and HT+Epo) as the other column (Fisher's exact test) using SPSS (SPSS Inc., Chicago, Ill., USA). All other comparisons were made using univariate ANOVA analysis with fixed factors of asphyxia (yes or no) and treatment (saline, HT, and HT+Epo) with appropriate post hoc comparisons to UCO saline using SPSS.

Results

Fifty-six *M. nemestrina* nonhuman primates were included in this study. Forty-two were delivered after UCO for either 15 (n = 19) or 18 min (n = 23) and randomized to: saline (n = 14), Epo (n = 6), HT (n = 10), or HT+Epo (n = 12). Controls (n = 14 total) included age-matched surgically delivered animals (n = 9) and concurrent colony controls (n = 5). Two of the 6 Epo-only-treated animals were disqualified: 1 due to a difficult resuscitation with prolonged hypoxia, and 1 due to a cardiac arrest while undergoing MRI. During the study period, HT became the clinical standard of care in humans. No further animals were enrolled in the Epo-only group, which excluded this group from analysis due to low numbers. Three additional animals were excluded from the final analysis: 1 animal randomized to HT (could not be resuscitated at birth) and 2 control animals (1 required intubation at birth with evidence of intrauterine compromise [29], and 1 had unexpected idiopathic hydrocephalus by MRI).

Of the 35 asphyxiated animals included in the analysis, 5 (14%) died prior to the planned necropsy, with deaths distributed by treatment as shown in figure 2. Three deaths occurred early (2 were euthanized at 3 days of age, and 1 at 6 days of age for severe neurological compromise), and 2 were due to late complications (1 died due to aspiration pneumonia, and 1 died unexpectedly at 5 months after unexplained tachypnea since the first week of age). Three deaths were in animals exposed to 15 min of UCO, and 2 were in animals exposed to 18 min of UCO. The gestational age of the study animals at birth was not different between controls and UCO animals (169 ± 1 vs. 168 ± 1 days, $p = 0.34$). Birth weight (547 ± 16 vs. 547 ± 13 g, $p = 0.99$), head circumference (20.6 ± 0.2 vs. 20.5 ± 0.1 cm, $p = 0.57$, and crown-rump length 20.6 ± 0.2 vs. 20.3 ± 0.1 cm, $p = 0.28$) were not different between groups. Epinephrine was given in 32% of resuscitations, and 20% of animals received sodium bicarbonate in the first 3 h after birth. The initial neurologic exam of all UCO animals prior to study treatments was profoundly abnormal, with no spontaneous respirations or movement, no withdrawal to painful stimuli, and flaccidity. All UCO animals required intubation and mechanical ventilation before study treatments were administered. UCO animals differed from controls in initial pH (6.94 ± 0.03 vs. 7.19 ± 0.04 , $p < 0.001$), base deficit (19.4 ± 0.7 vs. 9.3 ± 1.3 , $p < 0.001$), and serum lactate (13.2 ± 0.4 vs. 5.9 ± 0.9 , $p < 0.001$). The Apgar scores in animals with UCO of 0, 15, and 18 min are shown in figure 3. An early aEEG

in all UCO animals was consistent with the obtunded physical exam, with lower margins generally $<2 \mu\text{V}$, upper margins $<10 \mu\text{V}$, and a burst suppression pattern. A slow improvement in voltage occurred over the first 6 h of life. Phenobarbital was given to 49% of UCO infants for documented clinical seizures (7 UCO saline, 5 HT, and 5 HT+Epo; Pearson's χ^2 , $p = 0.81$).

Growth

Figure 4 illustrates the daily weights of animals over 38 postnatal weeks to show that the average weights for UCO-exposed animals remained below the 50th percentile compared to control animals despite adjustments to the caloric density of formula (up to 24 kcal/ounce) to foster weight gain. The inset in figure 4 is a comparison of the mean time to regain birth weight and shows that this time was increased for both the UCO saline and the UCO HT groups. UCO was associated with a decreased head circumference growth rate ($p < 0.05$), and this parameter improved with all treatments (data not shown).

Behavioral Results

UCO was associated with delayed achievement of early developmental milestones, including the ability to regulate temperature, as reflected by the number of days required to wean from an incubator and maintain euthermia without a heating pad (table 1). UCO also delayed the animals' ability to feed themselves (table 1). The time to resolution of primitive reflexes (rooting, righting, snout, sucking, and placing) was also delayed by UCO (table 1). Study treatments did not significantly affect these parameters; however, infants treated with HT+EPO trended towards improved scores. Compared to controls, UCO animals exhibited delays in multiple behavioral indices including the capacity to: sit, stand, walk, climb, and climb off a diaper roll (MRT); leave the security of their 'sleep diaper' in order to explore the playroom (diaper trip); release their grasp and explore when challenged to hang from a support (release grasp); touch another animal (initiate contact), and climb up a chain (table 2). Concurrent HT+Epo treatment significantly decreased the age at which infants reached for a toy, exhibited hand-over-hand chain climbing (table 2) and object permanence (table 3) compared to UCO saline-treated infants.

The visual paired-comparison test methodology was used to study emerging recognition memory skills in the first months of postnatal life [for review, see 30]. On problems using simple geometric forms, UCO saline infants performed poorly, while infants receiving HT+Epo provided significant evidence of recognition memory ($p <$

Fig. 2. Death or severity of CP after UCO. Number of animals with death or moderate/severe CP (top). Individual severity scores for all animals by treatment group (bottom). The severity of CP was based upon 4 physical therapy exams performed at 1 week and at 1, 4, and 8 months of age. UCO increased the rate of death or moderate/severe CP as compared to controls (Pearson's χ^2 * $p < 0.05$). Treatment with HT+Epo improved outcomes (Dunnett's test, $\dagger$ $p < 0.05$).

Fig. 3. Initial response to UCO. Data are mean ($\pm$ SEM) Apgar scores for neonatal animals separated by the duration of UCO (Pearson's χ^2 * $p < 0.05$).

Table 1. Neonatal behavioral measures

Index	Controls	UCO all	UCO saline	UCO HT	UCO HT+Epo
Animals, n	12	32	12	8	12
Out of the incubator	5.4±0.4	9.7±0.8*	11.0±2.2	9.6±0.5	8.5±0.4
Off the heating pad	11.8±0.4	16.1±1.0*	17.7±2.5	15.6±0.8	183.0±0.8
Self-feeding	12.7±0.6	23.3±2.7*	27.6±4.0	28.5±8.6	16.5±0.8
Primitive reflexes	2.5±1.6	5.7±0.7*	7.4±1.1	5.5±1.5	4.1±0.7

Data are the mean age (±SEM) at which a task was successfully performed for each separate treatment group unless otherwise stated. Significant ANOVA post hoc tests are indicated as * $p < 0.05$ compared to controls.

Table 2. Developmental behavioral measures

Index	Controls	UCO All	UCO saline	UCO HT	UCO HT+Epo
Animals, n	12	32	12	8	12
MRT	9.8±1.4	11.3±0.8*	13.5±0.4	11.0±1.9	9.4±1.5
Diaper trip	18.2±2.1	23.3±1.7*	27.1±2.1	22.4±4.5	20.1±2.3
Release grasp	41.5±4.0	54.2±6.3*	67.2±9.1	59.4±19.4	37.8±3.4
Initiate contact	19.1±2.4	25.8±1.7*	28.3±2.4	25.4±4.6	23.5±2.4
Climb up a chain	96.8±6.2	125.5±9.0*	149.7±13.7	132.5±24.9	96.6±4.5 [†]
Climb down a chain	96.8±6.2	126.7±8.9	152.6±13.2	132.5±24.9	97.0±4.6 [†]
Reach for a toy	27.3±2.9	29.5±1.9	34.3±2.9	31.4±4.1	23.6±2.3 [†]

Data are the mean age (±SEM) at which a task was successfully performed for each separate treatment group. Significant ANOVA post hoc tests are indicated as * $p < 0.05$ compared to controls, and [†] $p < 0.05$ compared to UCO saline.

Table 3. Object permanence

Index	Controls	UCO all	UCO saline	UCO HT	UCO HT+Epo
Animals, n	12	32	12	8	12
No screen	35.8±2.8	39.4±1.9*	45.3±2.5	38.4±4.1	34.3±3.0 [†]
With screen	36.2±2.8	42.1±2.3*	47.3±2.3	41.8±6.3	37.1±3.8
Item in well	38.3±2.5	40.4±2.2	47.1±2.8	38.9±4.1	34.7±3.8 [†]

Data are the mean age (±SEM) at which a hidden object was recognized for each separate treatment group. Significant ANOVA post hoc tests are indicated as * $p < 0.05$ compared to controls, and [†] $p < 0.05$ compared to UCO saline.

0.05). This finding suggests a disruption in the early memory processing of UCO infants, but also a sparing of some adverse memory effects in animals receiving treatment. When more challenging test problems were administered, both UCO saline and treated animals failed to provide evidence of memory when compared to control infants. Analysis of overall performance across experimental

groups, independent of problem difficulty, revealed that the highest memory scores were observed in control and HT+Epo infants ($p < 0.0001$). Animals in the HT treatment group were able to provide some evidence of memory on this task ($p < 0.05$), but control and HT+Epo infants provided the strongest psychometric profile on this early measure of cognition and information processing.

Fig. 4. Longitudinal growth of nonhuman primates. The main graph plots the average weight of UCO-exposed animals (lines) superimposed upon the individual daily weights of control animals (squares), with an inset graph plotting the mean ($\pm$ SEM) days to regain birth weight. The mean daily weights for the UCO saline (black line), UCO HT (blue line), and UCO HT+Epo (red line) groups are shown separately. The inset shows that the time to regain birth weight was significantly elevated for UCO saline and UCO HT animals (Dunnett's test, $^{\dagger} p < 0.01$). These graphs illustrate that UCO reduces the growth of animals despite caloric adjustments to foster weight gain.

MRI and Spectroscopy

No significant effects of UCO or treatment were detected on gross brain structure evaluated either early (days 1–3) or late (6 or 9 months). To evaluate brain growth, brain volume at 9 months was compared to brain volume within 72 h of birth ($n = 35$, 7 controls, 9 UCO saline, 6 HT, and 8 HT+Epo). There were no effects of UCO on total brain, cortical, or basal ganglia volumes. In contrast, cerebellar volume was decreased by UCO compared to controls, and combined HT+Epo treatment improved cerebellar growth after UCO (fig. 5). There were no left-right differences in brain volumes or specific MRI lesions among the study groups.

We used FA to evaluate fiber density, axonal diameter, and myelination. FSL tract-based spatial statistics revealed changes in the mode of anisotropy between UCO saline and control animals (fig. 6) [31]. FA, relative anisotropy (fig. 6), and volume ratio differences between HT+Epo and UCO saline were detected in animals scanned at 72 h. At 9 months, the raw T2 signal was increased in injured animals, with UCO saline animals having the highest signal and controls the lowest signal (fig. 4). In animals that underwent UCO for 18 min, ear-

Fig. 5. Cerebellar growth from birth to 9 months of age. Box-and-whisker plots (median and quartile range) illustrate the percentage increase in cerebellar volume for animals in each treatment group. Cerebellar growth was decreased by UCO compared to controls. Significant differences are indicated (ANOVA, $* p \leq 0.05$). Treatment with HT+Epo is associated with improved growth after UCO when compared to UCO saline-treated animals (Dunnett's test, $^{\dagger} p = 0.019$, $n = 9$ UCO saline, $n = 6$ UCO HT, and $n = 8$ UCO HT+Epo).

ly scans (24 and 72 h) of HT+Epo animals showed improved FA, relative anisotropy, and improved volume ratios compared to UCO saline animals (fig. 7). The mode of anisotropy differentiated between UCO saline and HT+Epo animals within the first 72 h after a UCO duration of 18 min. At 9 months, the raw T2 signal was similar to the combined 15- and 18-min UCO results.

Spectroscopy results are shown in table 4. The N-acetyl aspartate (NAA)/creatine ratio in the first 72 h and the GABA level at 9 months were altered by UCO. While the GABA levels decreased with treatment, this change was not statistically significant. Treatment did affect the choline level and the choline/creatine, NAA/choline, and NAA/creatine ratios so that animals treated with HT+Epo demonstrated improved values. Choline levels correlated with death or moderate-severe CP in animals that underwent UCO (Pearson's correlation 0.375, $p = 0.029$, $n = 34$). Analysis of phosphorus data did not reveal any effects of UCO or treatment. However, the overall energy available to cells increased (as measured by lower inorganic phosphorus levels) to control values over time in the HT and HT+Epo groups.

Fig. 6. DTI after UCO. TBSS analysis of mode of anisotropy, relative anisotropy, and raw T2 signal maps with red indicating areas that had an increased signal compared to UCO saline and blue areas indicating lower signals. Highlighted (red or blue) regions, $p \leq 0.05$ using threshold-free cluster enhancement with correction for multiple comparisons. Higher values of mode of anisotropy and relative anisotropy are associated with healthy white matter. Lower values of T2 signal are associated with healthy white matter. Top and middle: validation of the perinatal asphyxia model; bottom: demonstration that HT+Epo improves DTI.

Table 4. MRS findings

	Controls	UCO all	UCO saline	UCO HT	UCO HT+Epo
First 72 h					
Animals, n	8–12	27–34	11–13	7–9	9–12
Choline, mM/kg	1.60±0.05	1.47±0.04	1.63±0.07	1.44±0.03 [†]	1.32±0.05 [†]
Choline/creatine	0.29±0.01	0.27±0.01	0.30±0.01	0.26±0.01 [†]	0.25±0.01 [†]
NAA/choline	2.79±0.08	2.79±0.09	2.50±0.13	2.81±0.12	3.13±0.11 [†]
NAA/creatine	0.79±0.02	0.74±0.01*	0.73±0.02	0.72±0.03	0.78±0.02
9 months					
Animals, n	8	24	9	6	9
GABA, mM/kg	0.91±0.08	0.76±0.04*	0.67±0.08	0.80±0.05	0.82±0.08

Data are mean (±SEM) values determined by MRS at two different ages separated by treatment group.

Significant ANOVA post hoc tests are indicated as * $p < 0.05$ compared to controls, and [†] $p < 0.05$ compared to UCO saline.

Safety Parameters

Blood urea nitrogen levels were elevated 24 h after UCO compared to controls (20.7 ± 1.0 vs. 16.3 ± 2.4 mg/dl), but this did not persist, and no treatment effects were noted. Serum creatinine levels were not different between

control and UCO animals. Animals undergoing HT therapy had lower serum creatinine values at 24 h than did infants receiving UCO saline (UCO saline 1.20 ± 0.07 mg/dl; HT 0.96 ± 0.06 mg/dl, $p = 0.071$; HT+Epo 0.92 ± 0.08 mg/dl, $p = 0.017$). Liver function tests (aspartate amino-

Fig. 7. DTI after 18 min of UCO. TBSS analysis of FA, relative anisotropy (RA), and volume ratio (VR) between UCO saline and HT+Epo after 18 min of UCO is shown. DTI scans occurred within the first 72 h. Red areas indicate where the HT+Epo group had higher values than the UCO saline group, while blue signifies lower values in the HT+Epo group. Highlighted regions (red or blue), $p \leq 0.05$ using threshold-free cluster enhancement with correction for multiple comparisons. Colors are visible online only. Higher values of FA and relative anisotropy are indicative of healthy white matter. Lower values of volume ratio are indicative of healthy white matter.

transferase, alanine aminotransferase, serum bilirubin, and cholesterol) did not differ at any time point. Although not affected by treatment, white blood cell counts were decreased in UCO infants compared to control animals at 1 week of age ($8.2 \pm 1.1 \times 1,000/\mu\text{l}$ vs. $5.1 \pm 0.3 \times 1,000/\mu\text{l}$, $p = 0.003$) but not at any other time point (table 5). Hematocrit and platelet counts were unchanged by UCO or treatment group at any time point (table 5).

Epo Pharmacokinetics

The peak Epo concentration (C_{max}) for animals receiving 3,500 U/kg Epo+HT was $60,996 \pm 25,635$ mU/ml ($n = 6$) compared to $16,290 \pm 3,678$ mU/ml for those who received 1,000 U/kg Epo+HT ($n = 4$). Note that insufficient samples were available from some animals to conduct a complete pharmacokinetic analysis and that one of the higher-dose infants received 3,000 U/kg due to a dosage administration error. Of the 4 animals receiving 1,000 U/kg, 3 had no CP and 1 had mild CP. Of the 6 receiving

Table 5. Complete blood count results

Age, days	Control	UCO all	UCO saline	UCO HT	UCO HT+Epo
Hematocrit, %					
1	40.5 ± 1.4	42.4 ± 0.9	44.0 ± 1.3	41.1 ± 2.0	42.9 ± 1.5
7	35.0 ± 1.3	37.3 ± 0.8	37.1 ± 1.2	36.2 ± 1.0	38.4 ± 1.5
270	41.0 ± 1.3	40.4 ± 0.4	40.4 ± 0.7	40.5 ± 0.8	40.1 ± 0.6
Platelet count, $\times 1,000/\mu\text{l}$					
1	360 ± 31	326 ± 13	333 ± 26	295 ± 25	353 ± 26
7	512 ± 36	477 ± 28	501 ± 65	419 ± 25	498 ± 24
270	500 ± 22	462 ± 20	495 ± 33	404 ± 37	451 ± 38
White blood cell count, $\times 1,000/\mu\text{l}$					
1	7.6 ± 1.3	6.7 ± 0.4	7.5 ± 0.4	5.8 ± 0.8	7.0 ± 0.7
7	8.2 ± 1.1	$5.1 \pm 0.3^*$	5.2 ± 0.5	4.0 ± 1.2	5.6 ± 0.3
270	7.3 ± 1.2	5.8 ± 0.5	5.8 ± 0.6	6.0 ± 1.7	4.9 ± 0.3

Data are the mean values ($\pm$ SEM) of laboratory measures for separate treatment groups. UCO significantly lowered the white blood cell counts on day 7 compared to controls as indicated (ANOVA, $*p < 0.05$).

Fig. 8. Pharmacokinetics of Epo in the setting of HT. **a** Peak Epo concentrations ($C_{\max}$) of the 10 animals treated with HT+Epo divided into outcomes of no CP or mild CP. **b** AUC. **c** Mean ($\pm$ SEM) plasma concentrations of Epo in the first 72 h. The Epo concentrations of non-Epo-treated asphyxiated animals are shown by diamonds. The pharmacokinetics of high-dose Epo are depicted as circles, while the lower-dose Epo is shown as squares. Epo dosing occurred at 0, 24, and 48 h.

Table 6. Epo pharmacokinetics

Epo dose, U/kg	1,000	3,500
Animals, n	4	6
AUC, (U·h)/l	94,377 $\pm$ 5,801	413,948 $\pm$ 39,605
$C_{\max}$, U/l	16,290 $\pm$ 1,839	60,996 $\pm$ 10,465
Clearance, ml/h/kg	10.7 $\pm$ 0.7	8.6 $\pm$ 0.8
Half-life, h	6.1 $\pm$ 0.6	6.8 $\pm$ 1.0
MRT, h	7.2 $\pm$ 0.7	8.1 $\pm$ 0.5
V_D (steady state), ml/kg	76 $\pm$ 4	70 $\pm$ 6.9
V_D (area), ml/kg	93 $\pm$ 7	85 $\pm$ 15.1

Data are mean ($\pm$ SEM) values for 2 separate Epo doses. rEpo = Recombinant erythropoietin; V_D = volume of distribution.

higher-dose Epo, 2 had no CP and 4 had mild CP. Figure 8a and b shows the relationship of $C_{\max}$ and the AUC to outcome. The $C_{\max}$ in animals without CP was 31,889 mU/ml with a mean AUC of 215,520 U·h/ml. In comparison, the $C_{\max}$ for animals with mild CP was 54,337 mU/ml with a mean AUC of 356,719 U·h/ml. No animals receiving either dose of Epo in conjunction with HT died or had moderate or severe CP. A summary of all pharmacokinetic parameters is shown in table 6. As noted previously, Epo dosing results in nonlinear kinetics, with the higher dose resulting in a >3.5-fold increase in AUC [18, 32]. Of interest, endogenous Epo production was not increased by up to 18 min of UCO (fig. 8c).

Death and CP

In keeping with clinical trials of HT, our primary outcome was the presence of death (prior to planned necropsy) or CP (moderate-severe). Figure 2 shows the number of animals with early death or moderate-severe CP by treatment group. Outcomes for animals that underwent UCO were significantly different than control animal outcomes (Pearson's χ^2 , $p = 0.046$). Treatment changed the outcome of death or moderate-severe CP (Fisher's exact test, $p = 0.46$). Comparing the UCO saline and combined HT+Epo animals gave a relative risk reduction of 0.911 (95% CI -0.429 to 0.994), an absolute risk reduction of 0.395 (95% CI 0.072-0.635), and a number needed to treat of 2 (95% CI 2-14).

Discussion

In agreement with our prior report [15], 15-18 min of UCO in *M. nemestrina* produces features that accurately model human perinatal asphyxia, including an abnormal

physical exam, acidosis, elevated lactate, large base deficits, and Apgar scores that are all comparable to data from human infants enrolled into studies of moderate to severe HIE. Because HT is now the standard of care for the treatment of neonatal HIE [3], the safety and efficacy of new therapies for HIE must be evaluated in concert with HT. This study is the first to evaluate the safety and efficacy of Epo for perinatal asphyxia in combination with HT therapy in primates. The major findings of this study are that, although 72 h of HT alone did not reduce the risk of death or moderate-severe CP after UCO, HT combined with 4 doses of Epo decreased the risk of death or moderate-severe CP to 0% and preserved normal motor functions. The improvements in motor function are consistent with the finding that combined HT+Epo therapy also preserved cerebellar growth.

The optimal dose and regimen for human Epo neuroprotection is still not known. During the course of this study, we observed that peak Epo concentrations in hypothermic neonatal primates given 2,500 U/kg were 25% greater than expected when compared to data from normothermic infants given 2,500 U/kg [12] and, therefore, we lowered the Epo dose to 1,000 U/kg as a safety precaution. At 1,000 U/kg, the pharmacokinetic parameters obtained from hypothermic primates ($C_{\max} \sim 16,000$ U/l, AUC $\sim 94,000$) were comparable to those obtained from normothermic human neonates ($C_{\max} \sim 14,000$ U/l, AUC $\sim 81,000$) [18, 32]. This dose also results in pharmacokinetic parameters similar to those obtained with 5,000 U/kg in rats ($C_{\max}$ 6,000–10,000 U/l, AUC 120,000–140,000) [33]. Collectively, given the evidence in rodent models that 5,000 U/kg produces neuroprotection [13] and that multiple injections and late 1-week dosing are most effective [34–36], and given that no harmful drug effects have been noted clinically, we are satisfied that repeated 1,000 U/kg i.v. Epo is a safe and effective dose, suitable for future neuroprotection studies.

Several minor observations about the model deserve comment. The initial pH of the control animals was lower than what would be considered normal in humans, but it quickly normalized. Perhaps the lower pH is secondary to surgical delivery; general anesthesia or umbilical catheter placement produce brief HI and subsequent mild acidosis. As seen previously [15], UCO slightly reduced the growth of animals despite nutritional adjustments and it is possible that delayed somatic growth influenced neurodevelopmental outcomes. Also, it is interesting to note that endogenous Epo concentrations were not increased in control animals or in animals exposed to 15–18 min of UCO. Apparently, this duration of UCO is insufficient

to acutely induce HIF-1 and elevate endogenous Epo release. The lack of an endogenous neonatal response strengthens the rationale for early use of exogenous Epo in neonates after HI.

The DTI findings in this study changed over time after UCO, consistent with pseudonormalization as brain injury evolves [37]. Mode of anisotropy correlated with HI injury within the first 24 h, and we speculate that this index might be interesting as an early diagnostic biomarker of HIE. HT affected the natural progression of DTI changes after HIE. HT+Epo animals tended to have DTI results most similar to those of the controls. The elevated T2 signal in UCO saline animals is similar to the diffuse excessive high-signal intensity (DEHSI) seen in preterm infants; however, the neurodevelopmental outcomes of DEHSI are currently debated [38, 39].

The optimal timing of MRI following injury is variable, depending on whether maximizing sensitivity or specificity is the goal. The known timing of injury in this model provides an opportunity for further delineation of the best practice for timing of MRI as the brain injury evolves over time. MRS has been used to demonstrate injury to the brain during HI [40, 41]. We expected to see changes in several metabolites, especially the NAA/lactate ratio, but the only differences between controls and UCO were the NAA/creatine ratio in the first 72 h and GABA at 9 months. However, HT and HT+EPO decreased the amount of choline and resulted in better ratios of choline/creatine and NAA/choline. Increased choline levels have been correlated with an increased risk of mortality following HIE [42]. HT+EPO treatment had no deaths and had the lowest choline levels. Choline may be a biomarker of response to treatment and/or a prognostic biomarker. We speculate that choline might be used to determine the length of HT to improve outcomes.

Cerebellar hypoplasia is seen in human infants after HIE [43]. UCO saline decreased the rate of growth in the cerebellum, and treatment with HT+Epo normalized cerebellar growth. The motor problems associated with cerebellar abnormalities are well documented, but there is mounting evidence that the cerebellum is also involved in cognitive performance and social interactions [44, 45]. Poor neurocognitive outcomes of cerebellar injury/hypoplasia are increasingly being seen in preterm infants without cortical injuries [46]. Social interaction is impaired in humans with autism, and autopsy reports show that 95% of autistic children have hypoplastic cerebella [47]. While some of the animals that underwent UCO manifested autistic-like repetitive behaviors like self-clasping and rocking, no formal testing was done in this cohort.

This model of UCO has several strengths. Brain development and complexity are similar to those in humans, allowing for complex neurocognitive testing over time [48]. This study measured developmental indices until 9 months of age, which is comparable to 3 years of human development. The clinical and laboratory findings of asphyxiated animals are very similar to those in humans with moderate to severe HIE. Unlike clinical scenarios in human neonates, however, the timing and severity of the initial injury are known in this model, allowing for uniform initiation of treatments across groups [49–52]. This allows for detection of biomarkers that differentiate the timing and severity of injury.

Limitations of this study include the small number of animals in each group, the refinement of dosing regimens (discussed above), and the change from 15 to 18 min of UCO. This increase in UCO duration was effected because, as developmental and long-term MRI data were collected, it became apparent that 15 min of UCO was insufficient to reliably produce the significant disability and lasting brain injury needed to test neuroprotective strategies. The repeated developmental testing may also have served as unintended physical therapy, fostering improved outcomes. Work with nonhuman primates requires teams of clinicians and technicians, sophisticated diagnostic procedures, intensive 24-hour care and longitudinal behavioral testing, so large studies can be prohib-

itively expensive. Despite the small number of animals, we are confident that the data presented is informative. Like their human counterparts, these animals exhibit variability in the degree of brain injury sustained, providing an excellent opportunity to identify potential biomarkers of the severity of brain injury, response to therapy, and prognosis.

In conclusion, HT+Epo improved survival without moderate-severe CP, preserved cerebellar growth rates, and improved many neurocognitive behavioral scores after perinatal asphyxia. There were no adverse events attributable to Epo, suggesting that Epo use is safe in the setting of HIE and HT. This translational study sets the stage for further studies of HT+Epo in human neonates with HIE. Future treatment for HIE will likely be multimodal, with accumulating data advocating that Epo be included as one of these treatments.

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