Defining Longer-Term Outcomes in an Ovine Model of Moderate Perinatal Hypoxia-Ischemia

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Abstract
Hypoxic-ischemic encephalopathy (HIE) is the leading cause of neonatal morbidity and mortality worldwide. Approximately 1 million infants born with HIE each year survive with cerebral palsy and/or serious cognitive disabilities. While infants born with mild and severe HIE frequently result in predictable outcomes, infants born with moderate HIE exhibit variable outcomes that are highly unpredictable. Here, we describe an umbilical cord occlusion (UCO) model of moderate HIE with a 6-day follow-up. Near-term lambs (n = 27) were resuscitated after the induction of 5 min of asystole. Following recovery, lambs were assessed to define neurodevelopmental outcomes. At the end of this period, lambs were euthanized, and brains were harvested for histological analysis. Compared with prior models that typically follow lambs for 3 days, the observation of neurobehavioral outcomes for 6 days enabled identification of animals that recover significant neurological function. Approximately 35% of lambs exhibited severe motor deficits throughout the entirety of the 6-day course and, in the most severely affected lambs, developed spastic diparesis similar to that observed in infants who...
survive severe neonatal HIE (severe, UCOs). Importantly, and similar to outcomes in human neonates, while initially developing significant acidosis and encephalopathy, the remainder of the lambs in this model recovered normal motor activity and exhibited normal neurodevelopmental outcomes by 6 days of life (improved, UCOi). The UCOs group exhibited gliosis and inflammation in both white and gray matters, oligodendrocyte loss, neuronal loss, and cellular death in the hippocampus and cingulate cortex. While the UCOi group exhibited more cellular death and gliosis in the parasagittal cortex, they demonstrated more preserved white matter markers, along with reduced markers of inflammation and lower cellular death and neuronal loss in Ca3 of the hippocampus compared with UCOs lambs. Our large animal model of moderate HIE with prolonged follow-up will help further define pathophysiologic drivers of brain injury while enabling identification of predictive biomarkers that correlate with disease outcomes and ultimately help support development of therapeutic approaches to this challenging clinical scenario.

Introduction

Neonatal hypoxic-ischemic encephalopathy (HIE) remains the leading cause of neonatal morbidity and mortality worldwide (∼4 million neonates annually) and accounts for nearly 25% of neonatal deaths (∼1 million newborns) [1–4]. Approximately 1 million infants per year survive with cerebral palsy and/or serious cognitive and other developmental disabilities [5, 6]. Furthermore, the global nature of the ischemia associated with HIE is responsible for the impairment of several different organ systems (central nervous system 28%, cardiovascular system 25%, kidneys 50%, and lungs 23%), resulting in significant additional morbidity [3]. Unfortunately, approximately 47% of affected infants that undergo therapeutic hypothermia (TH) are still at risk of death or serious disability [7]. While TH results in a reduction in brain injury and mortality following birth asphyxia in moderate to severe HIE [8], it is the only known treatment, the benefits are modest, and its use can be potentially harmful [9, 10]. The most commonly used grading system for infants born with HIE remains the Sarnat score, with infants graded as mild, moderate, or severe depending on their clinical signs [11]. The approximate breakdown tends to be mild (39%), moderate (39%), and severe (22%) [6]. The management and outcome vary significantly with grade of HIE. While infants born with mild and severe HIE frequently result in predictable outcomes, infants born with moderate HIE exhibit variable outcomes that are highly unpredictable [12]. Infants born with mild HIE are not typically offered TH. The ultimate goal of this study is to develop a large animal model with high translational potential to identify predictive biomarkers and evaluate potential neurotherapeutic agents to improve neurodevelopmental outcomes in the setting of HIE.

Different animal models have their strengths and limitations, but in general they have greatly facilitated our understanding of the pathophysiology of neonatal HIE [13]. An ideal animal model of neonatal HIE is characterized by similarities between both human and animal models in terms of pathophysiology, phenotypic and histopathological characteristics, predictive biomarkers for course or prognosis, response to therapies, suitability for evaluation of drug safety or toxicity, cost-effectiveness, and ability to study long-term neurological outcomes [14]. Most commonly used rodent models are inexpensive and provide excellent insight into basic molecular mechanisms and long-term neurodevelopmental outcomes. However, there is significant variability between animals, as well as strains of species. The most commonly used Rice-Vannucci model leads to unilateral insult, while bilateral common carotid occlusion model has high mortality, with neither fully representing human neonatal HIE. Importantly, the rodent brain significantly differs from the human in both its size and level of cortical gyration and the small size of rodents does not allow for more precise neurophysiologic monitoring and assessment of multi-organ function after the insult compared to large animal models [15]. While larger rabbit models of in utero hypoxia-ischemia wherein uterine artery obstruction at 75% of gestational age reliably produce motor deficits in newborns [16], they do not recapitulate injury incurred during the dramatic physiologic changes accompanying the fetal-neonatal transition during birth. Nonhuman primate models, such as monkeys, or baeboons, while highly similar to human, do not fully reproduce the injury seen in human neonates, and their use is restricted by ethical concerns and high experimental costs [15].

Piglet and sheep models have been invaluable in providing critical insights regarding cerebral blood flow (CBF) and metabolism, delineating the drivers of neurological injury, or assessing responses to hypothermia after hypoxic-ischemic insults in newborns [15]. While existing (141–145 days) term models of umbilical cord occlusion (UCO) in sheep are highly robust in recapitulating the perinatal pathophysiology and neurological impair-

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ment observed in the setting of HIE, they typically describe neurological outcomes only up to 72 h after injury [17–19]. Longer-term outcomes are frequently needed in most animal models of brain injury to properly assess therapeutic responses. Along these lines, we predicted that extending the monitoring of neurological outcomes to 6 days in our term model of HIE would enable us to identify potentially differing neurodevelopmental outcomes [20]. Importantly, we have opted not to utilize the near-term (121–123 days) model of bilateral carotid artery occlusion previously used to develop TH, as we were primarily interested in defining the mechanisms giving rise to neurological injury as well as the pharmacology of putative therapeutic agents in the setting of the fetal-to-neonatal transition at term as this represents the most likely clinical use scenario globally [21].

Consistent with our prediction, here we describe two groups of animals with similar neurological deficits early after perinatal hypoxic-ischemic injury with significantly differing outcomes by day of life 6. We provide a hemodynamic, histological, and neurological comparison of these two groups in our model. Our large animal model of moderate HIE with prolonged follow-up will help further define pathophysiologic drivers of brain injury while enabling identification of predictive biomarkers that correlate with disease outcomes and ultimately help support development of therapeutic approaches to this challenging clinical scenario.

**Materials and Methods**

**Animals**

All animal research was approved by the University of California Davis Institutional Animal Care and Use Committee and was performed in accordance with the Guide for the Care and Use of Laboratory Animals. Sheep of both sexes were used. Study groups included UCO asphyxiated lambs (UCO), \( n = 16 \), and the group of sham-surgery lambs (control), \( n = 11 \), who were exposed to the same instrumentation as the experimental group but without UCO.

**Induction of HIE**

HIE was induced via UCO in near-term lambs (141–143 days gestation, term ~147–150 days; \( n = 11 \)). Time-dated pregnant ewes were fasted for 12–24 h prior to surgery. The ewes were induced with ketamine and propofol and anesthetized with isoflurane for surgery according to IACUC-approved Standard Operating Procedure, SC-20-112, “Sheep Anesthesia: Surgical Research Facility, H-Building at TRACS.” Briefly, a jugular catheter or peripheral venous catheter was placed and the ewe given 4 mg/kg slow push IV propofol and 1–5 mg/kg ketamine. After anesthetic induction and intubation, the ventral abdomen was shaved and cleaned. Immediately prior to surgery, the pregnant ewes underwent ultrasound imaging under general anesthesia to verify pregnancy. After ultrasound, the ewe was then transferred into the operating room where she was placed on a mechanical ventilator. Anesthesia was maintained with 1–5% isoflurane through the endotracheal tube (ETT). The ventral abdomen was then given a standard surgical scrub (using either betadine or chlorhexidine and alcohol), and the ewe was placed on maintenance IV fluids, usually 5–15 mL/kg/h. Oxygenation of the ewe was monitored with an \( O_2 \) saturation probe, and hemodynamics were monitored with a noninvasive blood pressure cuff. A midline incision along the ventral abdomen (6–10') was made and the uterus exposed. The ewe was given IV antibiotics (penicillin G potassium 10,000–20,000 units/kg and gentamicin 1–2 mg/kg). After exteriorization of the fetal head, the fetus was intubated with an appropriate sized cuffed ETT. The lungs were drained of fluid passively by gravity, and the ETT was plugged to prevent gas exchange during gasping. Venous and arterial catheters were placed in the jugular vein and carotid artery for hemodynamic monitoring, blood sampling, and drug administration. Asphyxia was induced by UCO until the onset of asystole. Lambs used as controls were instrumented in an identical fashion but were delivered and resuscitated immediately following clamping and cutting of the umbilical cord. The umbilical cord was cut, lamb was delivered to a radiant warmer, and following 5 min of asystole as assessed by invasive hemodynamic monitoring the lambs were resuscitated using neonatal resuscitation program guidelines with positive pressure ventilation with a fraction of inspired oxygen \( FI_2 \) of 1.0. Resuscitation was not initiated with room air as asystole, and the need for chest compressions was universal given the severity of the model; therefore, oxygen therapy was clinically indicated. After 30 s of ventilation, external chest compressions were initiated. Chest compressions continued for 60-s intervals before reassessing the heart rate, and these efforts continued for up to 15 min. Epinephrine (0.01 mg/kg) was administered intravenously if inadequate response to oxygen, ventilation, and chest compressions was noted after 60 s of asystole following initiation of ventilation. Additional boluses of epinephrine were given if animals were unresponsive to initial doses. Saline boluses were not provided as oxygen, epinephrine, and chest compressions were typically sufficient to restore adequate perfusion following return of spontaneous circulation (ROSC). During asystole as assessed by invasive hemodynamic monitoring the lambs were resuscitated using neonatal resuscitation program guidelines with positive pressure ventilation with a fraction of inspired oxygen \( FI_2 \) of 1.0. Resuscitation was not initiated with room air as asystole, and the need for chest compressions was universal given the severity of the model; therefore, oxygen therapy was clinically indicated. After 30 s of ventilation, external chest compressions were initiated. Chest compressions continued for 60-s intervals before reassessing the heart rate, and these efforts continued for up to 15 min. Epinephrine (0.01 mg/kg) was administered intravenously if inadequate response to oxygen, ventilation, and chest compressions was noted after 60 s of asystole following initiation of ventilation. Additional boluses of epinephrine were given if animals were unresponsive to initial doses. Saline boluses were not provided as oxygen, epinephrine, and chest compressions were typically sufficient to restore adequate perfusion following return of spontaneous circulation (ROSC). During aswell as following resuscitation, a ventilator provided ongoing mechanical ventilation. Assisted ventilation was decreased and then ceased when the lamb was spontaneously breathing >50% of the time and maintained a peripheral oxygen saturation >85% at an \( FI_2 \) of 0.21. No intravenous fluids were administered during or after resuscitation. After extubation, the lambs were gavage fed for the first 24–48 h. The lambs were assessed over a 6-day period to determine neurodevelopmental outcomes and euthanized on day 6 with an overdose of euthanasia solution (100 mg/kg pentobarbital sodium, Lethabarb®; Virbac Pty. Ltd., Peakhurst, NSW, Australia).

**Histology and Immunohistochemistry**

Following euthanasia on day 6, brains were flushed with 500 mL of phosphate-buffered saline (PBS) and perfused with 500 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were post-fixed in 4% paraformaldehyde overnight and transferred to 20% sucrose for 2 days and 30% sucrose till they sank (14 days). Brains were then flash frozen in 2-methyl butane on dry ice and stored at ~80°C. Coronal sections were cut on a
cryostat (12-μm-thick serial sections). Double immunofluorescence labeling was performed on brain sections that were defrosted and air dried at room temperature for 1 h. Following antigen retrieval in 10 mM citrate buffer (pH 6.0) for 10 min at 80°C and a PBS wash, sections were incubated in blocking solution (5% normal donkey serum, 0.4% Triton X-100 in PBS) for 1 h at RT. Primary antibody incubation was done overnight at 4°C with rabbit anti-GFAP (GFAP, 1:500, Z0334; Agilent); rabbit anti-CNPase (CNPase, 1:200, C5922; MilliporeSigma); mouse anti-NeuN for neurons (NeuN, 1:200, MAB377; MilliporeSigma), goat anti-Iba1 for microglia (Iba-1, 1:200, NB100-1028; Novus Biologicals), mouse anti-caspase-3 (Casp-3, 1:200, NB600-1235; Novus Biologicals), goat anti-oligodendrocyte transcription factor 2 for oligodendrocytes (Olig-2, 1:200 AF2418; Novus Biologicals), rat anti-myelin basic protein (MBP, 1:200, NB600-717; Novus Biologicals), and mouse anti-adenomatous polyposis coli protein clone CC-1 (CC-1, 1:100, OP800100UG; MilliporeSigma). After three 5-min PBS washes, sections were incubated for 1 h at RT with appropriate secondary antibodies: donkey anti-goat Alexa Fluor 647 (1:500, A21447; Thermo Fisher), donkey anti-mouse Alexa Fluor 568 (1:500, A10037; Thermo Fisher), donkey anti-rat Alexa Fluor 594 (1:500, A-21209; Thermo Fisher), and donkey anti-rabbit Alexa Fluor 488 (1:500, A21206; Thermo Fisher). For nuclear staining, sections were stained with 4,6-diamino-2-phenylindol for 5 min. Slides were then washed and coverslipped with ProLong Gold Antifade (P36930; Invitrogen). Images were taken with a Leica TCS SP5 Spectral Confocal Microscope.

Histopathological Analysis

To define the anatomical localization of the injury, we grossly evaluated all areas of the brain on sections corresponding to s.640, and 1,200 of Sheep Brain Atlas [22] at ×5 magnification. For final analysis, we chose the anatomical areas where injury was detected and compared these to control animals. Confocal-like Z-stacks (×25 oil objective, 10 μm thick, 1 μm Z step) were acquired using a Zeiss microscope equipped with the confocal-like Optigrid device and Volocity software (version 6.3, Improvision; Perkin Elmer, Waltham, MA, USA). Matching regions of interest in control animals acquired under the same conditions served as a control. Every brain had a control with no primary antibodies for staining. Image capturing (using Volocity software) and analysis using Imaris software (version 9.6.2.; Oxford Instruments America Inc., Pleasanton, CA, USA) to assess NeuN, Olig-2, and CC-1 cell counts and Iba-1, MBP, CNPase volumes, and Image J software (version 2.0.0.-rc-69/1.52p; National Institutes of Health, Bethesda, MD, USA) to manually count cleaved Casp-3 cells and Ca1/2, Ca3 NeuN-positive cells were done in a blinded manner. We measured the number of cells that express NeuN, cleaved Casp-3 and Ca1/2, Ca3 NeuN-positive cells were done in a blinded manner. We measured the number of cells that express NeuN, cleaved Casp-3, Olig-2, CC-1, and total volume of cell bodies and fibers expressing Iba-1, GFAP, MBP, and CNPase per field of view measuring 1,350 × 1,050 × 10 μm³ (1.4 × 10⁷ μm³).

Injury Scores

Brain injury scores were determined in coronal sections stained with Fluoro-Jade (FJ) using modified Bjorkman scale of histopathological scoring in a blinded manner [23]. Regions scored includ-
ed the following: cortex (Ctx), caudate (Caud), putamen (Put), thalamus (Th), and hippocampus (Hc). Scores were assigned for each region as follows: 0 = no injury, 1 = mild manifested by single-layer scattered neurons in Ctx or scattered single neurons in CP-THC, 2 = moderate involving 2 layers of neurons in one anatomical area of the Ctx, or a single focus of damaged neurons in Caud/Put/Th/Hc, 3 = severe involving >2 cortical layers or >2 foci of damaged neurons in Caud/Put/Th/Hc for a total score of 0–15.

**Neurological Outcomes**

Neurological outcomes were evaluated daily for 6 days. Our neurobehavioral assessment (Table 1) was based on observations previously conducted in sheep to monitor their well-being following birth [24, 25]. We assessed the time (days) taken to reach normal lamb behavioral milestones after birth (head lift and shake; use of front and hind limbs; first use of four legs; standing; feeding proficiency; walking) for a total score of 7 (Table 1). Lambs were
fed 2 oz every 4 h by tube the first day. Afterward, the lambs got fed 2–6 oz by bottle depending on size of lamb 4 times a day. If they were not able to bottle feed, they continued with tube feeding 2–4 oz 4 times a day with bottle attempts every day until they were euthanized. As a result of this assessment, lambs were assigned to two different categories, severe (UCOs) and improved (UCOi), depending on whether their clinical course improved over the course of the 6-day observation period to achieve developmental milestones comparable to controls (N) reflected by full score of 7 (UCOi) or remained severe (<3) (UCOs).

**Statistical Analysis**
Analyses were performed using Prism 7 (GraphPad Software, San Diego, CA, USA). All data are shown as mean ± standard error of measurement. Data were subjected to a normality test. The differences between two groups were assessed by t tests. Grouped data were analyzed using one-way and two-way analysis of variance and subsequently subjected to Smidak post hoc analyses, and differences were considered significant at p < 0.05. The data that did not pass the normality test were analyzed using Kruskal-Wallis test. The hemodynamic data were analyzed using grouped analysis of the individual group’s means for a specific timepoint. Comparisons were made between the groups with different neurobehavioral outcomes: UCO subjected with persistent motor deficits and encephalopathy (UCOs), UCO subjected with transient motor deficits and encephalopathy and complete recovery on day 6 (UCOi) and control group (N), and all injured animals (I) versus control group (N) (online suppl. material; for all online suppl. material, see www.karger.com/doi/10.1159/000525150).

**Results**

**Neurobehavioral Outcomes Identified Two Groups of UCO Lambs**
Neurodevelopmentally, on day 0, uninjured control animals were able to lift and move their heads, and feed and use all four limbs resulting in a stable gait within a few hours of birth (Fig. 1a–c). Their walk was brief and not completely coordinated. However, control lambs reached these neurodevelopmental milestones rapidly and exhibited a coordinated walk and run by day 1 after birth (Fig. 1b). A group of UCO lambs (n = 3) achieved full neurodevelopmental milestones already on day 0, while the rest of the animals (n = 13) suffered from severe disability on days 0 and 1. This included an inability to use the hind limbs (n = 13), minimal use of the front limbs (n = 6), absent standing or walking (n = 13), and minimal head lift (n = 9) (Fig. 1b, d–f). However, for some animals a remarkable progress in neurodevelopmental outcomes evolved during our 6 days observation period (Fig. 1a, b). Almost half of the UCO animals (n = 7) with poor prior neurological function and total scores ranging from 0 to 2 on day 0 remarkably improved their motor function and achieved close to a full clinical recovery as early as day 2 after the UCO. A subgroup of UCO lambs with poor neurological score on day 0 improved to the full clinical score more slowly than those not as clinically impaired at this early timepoint, reaching full scores on days 4 (n = 1) and 5 (n = 1). A final subgroup of UCO animals (UCOs, n = 5) remained severely affected with scores ranging from 0 to 3, never achieving the ability to feed, or use their limbs, continued to have difficulties with feeding, and was minimally active (Fig. 1f). These animals also demonstrated increased motor tone and spasticity of the hind limbs. These findings of a movement disorder causing persistent activity limitation attributed to disturbances that occurred in the developing fetal brain, often accompanied by disturbances of behavior and secondary mus-
Fig. 3. ABG parameters: all animals have comparable BSN values. UCO leads to profound respiratory and metabolic acidosis reflected by pH < 7 (a), lower BE (b), high pCO₂ (c), hypoxia (d), and hyperlactatemia (e). f UCO also causes glc variability, with profound hypoglycemia immediately following resuscitation, followed by prolonged hyperglycemia. The resuscitation leads to brief period of hyperoxia with high pO₂. BSN, baseline; CPR, cardiopulmonary resuscitation; glc, glucose; BE, base excess; lac, lactate.
closkeletal problems, are consistent with clinical findings in infants with severe HIE (Fig. 1a–b, d–f) [19]. The distribution of outcomes in this model is thus consistent with the variation of clinical outcomes observed globally. We did not detect significant differences in body weight (Fig. 1g) at birth and explant and brain weight (Fig. 1h) at the end of the 6-day period, nor in brain-to-body weight ratio (Fig. 1i) among any of the studied groups.

Hemodynamic Parameters

All lambs were hemodynamically monitored via invasive arterial catheters starting in utero prior to UCO. We assessed for changes in hemodynamic parameters continuously for a duration of up to 60 min post-delivery. UCO resulted in hypotension and bradycardia, ultimately resulting in pulseless electrical activity/ asystole within approximately 16.18 ± 1.099 min (Fig. 2). We did not observe differences in measured hemodynamic parameters (systolic and diastolic blood pressures) as well as mean arterial pressure between the UCOi and UCOs groups (Fig. 2). The onset of asystole, duration of cardiopulmonary resuscitation (CPR), and time to ROSC were comparable between UCOs and UCOi cohorts (asystole: UCOi 16.64 ± 1.65 vs. UCOs 15.33 ± 0.84 min; CPR: UCOi 1.81 ± 0.18 vs. UCOs 1.66 ± 0.33 min; ROSC: UCOi 6.81 ± 0.18 vs. UCOi 6.66 ± 0.33 min). All animals received one dose of epinephrine. Two second doses of epinephrine were necessary to achieve ROSC in UCOi animals (2 of n = 11), while one second dose was given to one UCOs animal (1 of n = 5). No additional medications were administered.

Blood Gas and Glucose Analyses

Blood sampling to assess arterial blood gas (ABG) was undertaken in utero prior to UCO to assess baseline (BSN) status, at the end of asphyxia just prior to resuscitation (BSN), and post-resuscitation at regular intervals. ABG analysis revealed similar BSN parameters (pH, PCO₂, PO₂, glucose (glc), lactate (lac), bicarbonate, hemoglobin) for all groups (Fig. 3a–e; Table 2, BSN). UCO led to significant acidosis in injured lambs when compared with uninjured controls (Fig. 3a, b, e; Table 2, CPR, 10 min). Between the 2 clinically different groups of the UCO lambs (UCOs vs. UCOi), in the ones with more severe clinical outcome (UCOs) we noticed more significant metabolic acidosis reflected as lower pH (p = 0.03), bicarbonate (p = 0.02) (data not shown), and more profound base deficit during the CPR (p = 0.01). Both groups continued to have significant base deficit during the whole observed post-resuscitation period and hyperlactatemia at 60 min compared to control (Fig. 3b, e; Table 2, 10–60 min). While the UCOi animals had significant hypercarbia compared to controls 20 min after the UCO (p = 0.003), profound hypercarbia developed in the UCOs animals at 30 min (p < 0.0001) (Fig. 3c; Table 2, 20–60 min). The UCOs and UCOi animals also developed significant hyperoxia compared to controls (UCOs: p = 0.009, UCOi: p = 0.04) (Fig. 3d; Table 2, 10 min). UCO led to significant hypoglycemia in both UCOs and UCOi detected at 10 (UCOs: p = 0.006; UCOi: p = 0.006) and 20 min (UCOs: p = 0.03; UCOi: p = 0.01) after the insult (Fig. 3f; Table 2, 10–30 min). The hemoglobin levels were not significantly different in any of the studied groups (data not shown).

Histopathological Injury

Periventricular and Subcortical White Matter

Given the significant motor deficits observed, we initially evaluated brains for evidence of white matter injury. White matter injury was assessed by measuring the quantity and integrity of the major structural components of the myelin sheath including MBP, CNPase, as well as by the quantity of mature oligodendrocytes stained with CC-1 and total oligodendrocytes labeled by Olig-2. The overall cellularity evaluated by DAPI nuclear counts within periventricular white matter (PVWM), subcortical white matter of the cingulate gyrus (SCWM1), and subcortical white matter of the first parasagittal gyrus (SCWM2) was similar across all groups (data not shown).

MBP volume was significantly lower in PVWM and SCWM1 of the UCOs groups compared with control (PVWM: p = 0.002; SCWM1: p < 0.0001) (Fig. 4a). HIE-affected lambs in the UCOi group, however, had lower MBP volume versus control only in SCWM1 (p < 0.006) (Fig. 4a). The UCOs additionally had lower MBP volumes compared to the UCOi in PVWM (p = 0.002) and SCWM2 (p = 0.02). The myelin proteins stained by CNPase were unchanged in all groups compared with control (PVWM: UCOi, p = 0.75; UCOs, p > 0.99; SCWM1: UCOi, p > 0.99; UCOs, p > 0.99; SCWM2: UCOi, p = 0.91; UCOs, p = 0.54) (Fig. 4b).

Olig-2 staining of oligodendrocyte lineage cells was significantly increased in the UCOi group compared with control only in SCWM2 (p = 0.03) (Fig. 4c). Olig-2-positive cell counts differed between UCOs and UCOi groups in SCWM1 (p = 0.03) and SCWM2 (p = 0.01). The number of mature oligodendrocytes labeled by CC-1 marker did not significantly differ among studied groups (Fig. 4d).

Inflammation of the white matter was investigated by volumetric and morphologic assessment of astroglial...
Table 2. Blood gas analysis at different timepoints after the UCO

|          | BSN N | UCOs | UCOI | CPR N | UCOs | UCOI | 10 min N | UCOs | UCOI | 20 min N | UCOs | UCOI | 30 min N | UCOs | UCOI | 60 min N | UCOs | UCOI |
|----------|-------|------|------|-------|------|------|----------|------|------|----------|------|------|----------|------|------|----------|------|------|----------|------|------|
| pH       | 7.25± | 7.19±| 7.26±| 0.01  | 0.03 | 0.02 | 0.03     | 0.03 | 0.02 | 0.04     | 0.07 | 0.04 | 0.03     | 0.05 | 0.04 | 0.03     | 0.07 | 0.06 |
| pO2      | 21.83±| 22.28±| 21.99±| 0.48± | 0.36± | 0.28 | 0.18     | 18.96 | 16.04 | 7.00     | 28.30 | 20.85 | 10.88    | 24.67 | 6.47 | 5.92     | 15.77 | 11.38 |
| pCO2     | 61.13±| 60.8± | 61.69±| 0.48± | 0.36± | 0.28 | 0.18     | 18.96 | 16.04 | 7.00     | 28.30 | 20.85 | 10.88    | 24.67 | 6.47 | 5.92     | 15.77 | 11.38 |
| BE       | 0.08± | 0.86± | 4.68± | -8.46±| -13.42±| -10.13 | -10.13 | -10.13 | -10.13 | -10.13 | -10.13 | -10.13 | -10.13 | -10.13 | -10.13 | -10.13 | -10.13 | -10.13 |
| mmol/L   | 1.12  | 1.05  | 1.00  | 1.03  | 1.35  | 1.61  | 0.89     | 1.02  | 0.97  | 1.23     | 0.85  | 0.82  | 1.62     | 1.02  | 3.44 |
| Lac      | 2.12± | 3.54± | 1.87± | 8.90± | 7.45± | 2.29± | 9.76±    | 8.37± | 2.83± | 8.88±    | 7.87± | 2.60± | 8.66±    | 7.66± | 5.42± |
| mmol/L   | 0.33  | 0.91  | 0.29  | 0.72  | 0.32  | 0.66  | 1.20     | 0.29  | 0.84  | 0.98     | 0.28  | 0.69  | 0.99     | 0.21  | 0.73  | 1.10     | 0.30  |
| G1c      | 14.5± | 17.00±| 10.73±| 10.00±| 0.00± | 0.00± | 13.38±   | 8.12± | 6.42± | 40.75±   | 79.40±| 158.0±| 58.25±   | 77.22±| 67.00±| 44.67±   | 60.60±| 48.82±|
| mg/dL    | 2.4   | 3.96  | 0.89  | 0.72  | 0.32  | 0.66  | 1.20     | 0.29  | 0.84  | 0.98     | 0.28  | 0.69  | 0.99     | 0.21  | 0.73  | 1.10     | 0.30  |

Significance is as follows: *p* < 0.05, **p** < 0.01, ***p*** < 0.001, ****p**** < 0.0001.

BE, base excess; lac, lactate; glc, glucose; BSN, baseline; CPR, cardiopulmonary resuscitation. UCOs, severe, group with poor outcomes; UCOi-improved, group exposed to UCO insult with full neurological recovery at day 6; N, control group.
The neuroinflammation was quantified by total volume of Iba-1-positive microglial cells (e) and presence of gliosis (f). The cellular death was quantified by the number of Casp-3 positive cells. Brackets show significances as follows: * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \), \( p < 0.0001 \). UCOs (black) – injured animals with poor outcomes, UCOi (dark gray) – injured animals with improved outcomes on day 6, N (green) – controls, I (light gray) – all injured animals, I = UCOs + UCOi.

Gray Matter Injury

Histological injury was initially detected by using the FJ marker to identify degenerating neurons in brains on day 6. We observed degenerating neurons primarily in the cingulate gyrus (Ctx1) (Fig. 6a), extending to the parasagittal 1st (Ctx2) and 2nd gyri (Fig. 6b, c). In the Ctx, we detected damage to neurons in the II layer (Fig. 6d, e), more extensive injury involved neurons in the III–V layer (Fig. 6f), as well as deeper brain structures, such as Caud, Put, Hc, and Th (Fig. 6g–j). The extent of neuronal damage detected by FJ staining did not necessarily correlate with clinical outcomes, as some of the animals showing more extensive injury by FJ were clinically unaffected (Fig. 6k). However, the clinically most severely affected animals all exhibited neurological injury within the Ctx1/Ctx2 \( (n = 5) \), as well as severe damage to Hc \( (n = 3) \).

We assessed the extent of global neuronal loss on day 6 by measuring the total number of NeuN-positive neurons in Ctx1, Ctx2, Caud, Put, and Hc Ca1/2, Ca3. The total number of NeuN-positive neurons was lower in the Ctx of the cingulate gyrus and Ctx1 in UCOs animals compared to controls \( (p = 0.03) \), as well as the UCOi animals \( (p = 0.002) \). We did not detect significant changes in Ctx of the 1st parasagittal gyrus (Ctx2), nor in Caud or
Put in all groups studied (Fig. 7a). In Hc, the neuronal loss was significant in Ca1/2 in the UCOi group ($p = 0.03$), while the Ca3 region demonstrated fewer NeuN cells in the UCOs group compared to controls ($p = 0.01$) as well as compared to the UCOi group ($p = 0.01$) (Fig. 7a). The overall cellularity assessed by DAPI staining was comparable in all areas studied but Ctx2, where UCOs animals had higher number of DAPI-positive cells compared to controls ($p = 0.02$, data not shown).

We further assessed for apoptotic cell death by counting the total number of Casp-3-positive cells. In the Ctx dimension, and thickness of processes in all studied white matter areas in UCOs animals. Similarly, GFAP-stained astrocytes show thicker bodies and shortened processes. The Casp-3 is elevated in injured animals compared to controls (scale marker = 100 μm; blue – DAPI nuclear staining; the pathologies are pointed at by an arrowhead). PVWM, periventricular white matter; SCWM1, subcortical white matter of cingulate gyrus; SCWM2, subcortical white matter of the 1st parasagittal gyrus.

Fig. 5. Histological findings in white matter after UCO: representative photomicrographs of MBP, CNPase, Olig-2, Iba-1, GFAP, cleaved Casp-3, and CC-1 in PVWM, SCWM1, SCWM2. MBP shows reduction in the number of myelin fibers and myelin breaks in the injured UCOs brains compared to controls. The Olig-2 is elevated in SCWM2 in UCOi animals. The CNPase does not show significant changes in injured animals compared to the controls. The Iba-1 marker is elevated due to increased number, cell body
had elevation in Iba-1-positive cell volume only in the cortical area (Ctx1: \( p = 0.03 \), Ctx2: \( p = 0.02 \)) (Fig. 7c).

We did not observe changes in astrogliosis in either Ctx1, Ctx2, Caud, or Put gyrus among all groups studied (Fig. 7d). Increased astrogliosis was noticed in Ca1/2 when we compared all injured animals to control group (\( p = 0.02 \)); however, no significant differences were noted between groups UCOs (\( p = 0.06 \)) or UCOi (\( p = 0.94 \)) lambs. In Ca3 of the Hc, astrogliosis was observed in the UCOs animals compared to controls (\( p = 0.01 \)) (Fig. 7d). The focal injury site was characterized by shrunken, pyknotic NeuN\(^+\) cells, and neuronal loss (Fig. 7e, 8). Microglial cells at the injury site had ameboid features with enlarged cell bodies and fewer processes. The injury site exhibited accumulation of GFAP-positive fibers suggestive of gliosis (Fig. 7e, 8).

**Discussion**

We have identified two subgroups of animals that differ in their physiologic, histologic, and neurodevelopmental responses after suffering similarly severe metabolic insults observed in the term neonate suffering perinatal asphyxia. The UCO-affected lambs developed a severe metabolic acidosis prior to resuscitation similar to infants born with profound HIE, and all lambs exhibited significant encephalopathy typically lasting hours to days. Thirty-five percent of lambs exhibited severe motor deficits compromising ambulation throughout the 6-day course and, in the most severely affected lambs, exhibited spastic diparesis similar to that observed in older infants with severe HIE. The UCOs group exhibited gliosis and in-
Fig. 7. Histological changes in gray matter: quantitative changes in neuronal counts (NeuN, a), cellular death markers (Casp-3, b), and inflammatory markers of microglial accumulation (Iba-1, c) and gliosis (GFAP, d) in cingulate gyrus Ctx (Ctx1), 1st parasagittal gyrus Ctx (Ctx2), Caud, Put, Ca1/2 and Ca3 of the Hc (Ca1/2 and Ca3). Brackets show significances as follows: *p < 0.05, **p < 0.01, ***p < 0.001. Representative photomicrographs of Ca3 area of injured Hc (e) show loss of neurons (NeuN-UCO) with more gliosis (GFAP-UCO), accumulation of activated amoeboid microglia (Iba-1-UCO), and significant cellular death (Casp-3-UCO) yellow arrowheads. The Hc in control brain shows normal neuronal structure (NeuN-control), less gliosis (GFAP-control), ramified microglia (Iba-1-control), and lower counts of Casp-3-positive cells (Casp-3 control); white arrowheads. Scale marker = 100 μm; blue – DAPI nuclear staining.

flammation in both white and gray matters, oligodendrocyte loss, and neuronal loss and cellular death in the Hc and cingulate Ctx. The UCOI group showed more cellular death and gliosis in the parasagittal Ctx but demonstrated greater preservation of white matter markers, reduced markers of inflammation, and lower cellular death and neuronal loss in Ca3 of the Hc compared with UCOS lambs.

A common pathophysiologic pathway ultimately resulting in perinatal brain injury in humans is characterized by the sequence of hypotension with or without asystole, leading to primary energy failure due to brain hypoxia and ischemia followed by secondary energy failure due to reperfusion injury [26, 27]. Our sheep model met the commonly used clinical criteria that define an acute intrapartum event sufficient to cause HIE, including severe intrapartum acidosis with pH < 7 and base deficit >12 mmol/L, moderate/severe encephalopathy, spastic di/quadruplegia or dyskinesia, and absence of other factors [28]. Prolonged UCO in the near-term lamb led to a
double-insult consisting of profound hypotension and hypoxemia, and both needed to create an injury as near-term lambs have more mature CBF autoregulation [29]. Utilizing this model, we identified two subsets of animals in the UCO group that significantly differed in their clinical neurological outcomes despite suffering the same insult that led to a comparable change in hemodynamic parameters. The differences in neurological outcomes became evident only 3–6 days after injury. Approximately a third of the animals suffered severe neurological disability resulting in severe encephalopathy and spastic diplegia, while the second subgroup of injured animals improved their mental status and motor outcomes starting on day 3. The neurobehavioral outcomes in the latter group of animals fully matched the uninjured control group at day 6 after the insult. These two subgroups differed, however, in their biochemical parameters, as well as histopathological findings.

Blood gas analysis correlated with neurological outcomes, as the group of animals with more severe neurological injury had more profound acidosis with lower pH and more severe base deficits early during resuscitation. This is similar to near-term ovine UCO studies and human studies where pH, lac, and base deficit belong to the strongest predictors of clinical outcomes [17, 30]. These predictors also include the need for resuscitation in babies; however in our study, both groups underwent CPR suggesting presence of other factors impacting the out-

Fig. 8. Representative photomicrographs of histological changes in gray matter: images compare injured (UCO) versus control brains (control). Arrows point to glial cells with smaller processes and thicker cell bodies (GFAP), loss of NeuN cells with loss of organization of the Ctx (NeuN), accumulation of activated microglia with shorter processes, round cell bodies (Iba-1), and apoptotic cells with shrunken cell bodies and pycnotic nuclei suggesting cell death (Casp-3). Ctx, cortex; Caud, caudate; Put, putamen (scale marker = 100 μm; blue – DAPI nuclear staining).
comes. Similar to selected human studies, the lac levels took longer to normalize in the more severely injured animals which could be related to the degree of end-organ dysfunction impacting lac clearance or seizure activity and may predict outcomes [31, 32]. The impact of lac could be level-dependent, Da Silva observed that plasma lac concentration greater than 9 mmol/L was associated with moderate or severe encephalopathy with a sensitivity of 84% and a specificity of 67%, while lac < 5 mmol/L did not lead to significant encephalopathy [33]. The hemodynamic parameters were largely similar between the two injured groups. Notable also is hyperoxia at 10 min in both UCO groups after asystole resulting from the use of 100% O₂ for initial resuscitation. Hyperoxia in the immediate postnatal period has been associated with the increased incidence of HIE in term infants [31, 34]. However, as the hyperoxia is noticed in both groups, we exclude it as a major contributing factor responsible for different clinical neurological outcomes between the UCO groups in our model. The UCO insult was associated with a profound systemic hypoglycemia in injured animals at the time of CPR. Rapid depletion of glc that occurs during the hypoxic episode is associated with anaerobic metabolism, and rapid rise in lac in both hemispheres [35] and early hypoglycemia in term infants between 0 and 6 h is reported to be associated with worse outcomes [36]. The neonatal brain is especially susceptible to hypoglycemia compared to the adult brain [35, 37]. While treatment of the hypoglycemia after hypoxic-ischemic injury may reverse the anoxic vulnerability as shown by Vannucci and Vannucci [38], this phase is followed by an episode of hyperglycemia at 10–20 min after the UCO probably as a result of a stress response. Mallard et al. [39] observed hyperglycemia as early as 4 min at UCO; however, our measurements unfortunately did not include this timepoint. Hypoglycemia, hyperglycemia, and high glc variability are associated with poor neurological outcomes in term neonates [40–43]. However, the changes in glc were not significantly different between the more and less severe groups of UCO animals.

Our histological findings align with 2 major MRI patterns of term neonatal HIE described by Miller et al. [44]: watershed-predominant and basal ganglia/Th-predominant that are associated with different clinical presentations and neurodevelopmental outcomes [45]. Mild to moderate HIE produces parasagittal watershed zone infarcts involving both the Ctx and underlying subcortical white matter. Severe HIE results in injury to metabolically active tissues such as thalami, putamina, hippocampi, brainstem, corticospinal tracts, and sensorimotor Ctx [46]. Consistent with previous findings of white matter injury in near-term fetal sheep [47–49], our UCO model led to loss and disruption of myelination and increased numbers of microglia in the intragryral and PVWM of the more severely injured animals [50]. While decreased immunoreactivity in MBP with myelin breaks and areas of hypomyelination affected PVWM, SCWM1, and SCWM2 in the more injured animals, the CNPase stained myelin sheaths did not differ among groups. CNPase is considered an index of myelin formation where the amount of immunoreactive CNPase correlates with the thickness of the myelin sheath in the central nervous system [51]. Interestingly, a higher number of Olig2-positive cells were detected in SCWM2 in the less severely injured group. We did not detect significant loss of mature oligodendrocytes labeled by CC-1. Changes in white matter after brain injury are dynamic [52], and the quantitative description of white matter markers at 6 days after UCO likely reflects the process of an ongoing injury, as well as remyelination. We speculate that areas of unchanged oligodendrocyte count, MBP, and CNPase in less severe injured animals reflect some degree of remyelination that is less active in the group of animals with worse outcomes, where the MBP remains low, and CNPase and oligodendrocyte counts are unchanged.

Gray matter injury was initially assessed by using FJ staining that has been validated as a sensitive and reliable marker of ongoing neuronal degeneration [53, 54]. The FJ staining pattern was characterized by injury consistent with a combination of linear necrosis and focal patches. FJ staining revealed areas of specific neuronal susceptibility to the UCO that was located in almost all injured animals predominantly in the areas of the cingulate gyrus and parasagittal Ctx. The FJ-positive neurons were detected in striatum, Hc, and Th, although less frequently. This pattern of neuronal susceptibility is similar to other UCO studies in term sheep and term neonates after HIE [29, 39, 55]. The areas identified by FJ in our model correspond to areas of susceptibility to the regional CBF changes (parasagittal Ctx) [56] and areas of high glc utilization (cingulate Ctx, basal ganglia, and hippocampal regions) in neonates [57, 58]. Thus, if the compensatory mechanisms to maintain CBF and glc levels are exhausted, they lead to a characteristic injury pattern that is responsible for motor and cognitive deficits seen in neonates after HIE [44, 46]. While FJ enabled our study to distinguish between the injured and control animals, the overall FJ injury score did not predict clinical outcomes. This could be due to clearance of damaged neurons from the injury site at our chosen timepoint of 6 days. The Iba-1/NeuN/GFAP immunohistochemistries showed global...
changes, as well as detected focal injury patterns characterized by neuronal loss, microglial activation, and glial scar formation, an injury pattern described in nonhuman primates after severe HIE [59]. Neuronal loss detected by the NeuN marker was predominant in the parasagittal areas. Interestingly, cellular death at day 6 was more pronounced in the animals with better outcomes in Ctx. We speculate that this may be attributed to more active cellular turnover as a part of neuroregeneration. Significant cell death was found in Ca3 areas of Hc in the most severely injured animals, together with more profound neuronal loss, presence of microglial cells, and more astrogliosis. UCOs animals also demonstrated gliosis and microglial cell accumulation in Ca1/2; however, we did not detect significant neuronal loss. Combination of cell death and neuronal loss in Ca1/2 was notable in UCOi animals. This likely suggests more extensive inflammation in Hc and Ctx in UCOs animals with cellular loss. The neuronal loss more predominant in the Ca3 areas is similar to the observation of Mallard et al. [60]. Dense activated microglial infiltration notable in hippocampal stratum moleculare and radiatum suggests possible damage of hippocampal neuronal dendrites. Preserved interaction between pyramidal cells and interneurons in Hc is essential for learning and memory formation and for functional recovery after injury [61]; thus, excessive microglial activation could be one of the factors contributing to delayed hippocampal injury leading to worse neurobehavioral outcomes after HIE.

Our study has several limitations. While the birth weight, initial hemodynamic parameters, and biochemical data were similar among our groups, we cannot exclude other confounders to poor neurological outcomes in one group compared to the other. Defining additional physiological and biochemical factors that worsen outcomes in one group, or improve outcomes in the other animals, such as timing of the cord clamping or depth of chest compressions is crucial in understanding of the pathobiology of neonatal HIE [18], as well as for designing therapies. Because of the cost and maintenance of large animal studies, our study included lower numbers of animals representing individual groups than is typically accomplished with rodent studies. This prevented us from considering well-known effects of sex on response to brain HI and neurological outcomes [62, 63]. These factors also limited the duration of follow-up in these lambs. Our hemodynamic parameters did include only the first 60 min after UCO, where the vital signs did not typically return to the BSN preventing us from better defining the hemodynamic alterations. We did not assess subclinical seizure activity by EEG. The variability in severity of neurological injury poses a challenge to measure effectiveness of therapeutic interventions. This variability is also observed in clinical studies and is likely due to variation in intrinsic susceptibilities driven by genetic heterogeneity. Our use of an outbred ovine model thus accurately reflects the heterogeneity observed clinically. Our pathohistological investigation did not include staining for all cell types, regions, and processes relevant to HIE, including cerebellum, or different mechanisms of cell death. We plan on addressing these deficiencies in future studies.

It is well known that brain injury following HIE evolves over time, and some potential therapies show benefit even when administered outside of the initial 6-h window critical for TH [26]. Our large animal model of moderate HIE with prolonged follow-up will help further define pathophysiologic drivers of brain injury while enabling identification of predictive biomarkers that correlate with disease outcomes and ultimately help support development of therapeutic approaches to this challenging clinical scenario. Our findings thus suggest that assessing neurodevelopmental outcomes and the effects of neurotherapeutic interventions might need to be extended at last until 6 days after UCO in ovine models.

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Statement of Ethics

All animal research was approved by the University of California San Francisco Institutional Animal Care and Use Committee and was performed in accordance with the Guide for the Care and Use of Laboratory Animals. This study protocol was reviewed and approved by UC Davis IACUC, approval number 20777.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Jana K. Mike and Emin Maltepe: substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; drafting the article; and final approval of the version to be published. Praneeti Pathipati: substantial contributions to statistics. Christine Windsor, Katherine Y Wu, and Samuel Os- trin: substantial contributions to tissue processing and histologies. Janica Ha: substantial contributions to tissue processing, histologies, and data analysis. Blaise Ndjamé: substantial contributions to image processing and data analysis. Rachel S. Hutchings, Chris- tian Vento, and Oona Vanhatalo: substantial contributions to animal handling, tissue processing, and neurodevelopmental and he- modynamical data acquisition. Brian D. Goudy, Peggy Chen, and Ziad Alhassen: substantial contributions to animal handling. Courtney Losser and Kimberly Arellano: substantial contributions to animal surgery and handling. Payam Vali, Satyan Lakshmin- rusimha, Jogarao V.S. Gobburu, Janel Long-Boyle, and Yvonne W. Wu: substantial contribution to experimental design, data analy- sis, and interpretation. Yasmine White: substantial contributions to animal handling, data analysis, and interpretation of data. Jef- frey R. Fineman and Donna M. Ferriero: substantial contributions to neurodevelopmental data analysis and interpretation of data; drafting the article; and final approval of the version to be published.

Data Availability Statement

All data generated or analyzed during this study are included in this article and its online supplementary material. Further in- quiries can be directed to the corresponding author.

References

1. Lawn JE, Manandhar A, Haws RA, Darmstadt GL. Reducing one million child deaths from birth asphyxia: a survey of health systems gaps and priorities. Health Res Policy Syst. 2007 Dec;5(1):4.
2. Lawn JE, Lee AC, Kinney M, Sibley L, Carlo WA, Paul VK, et al. Two million intrapartum-related stillbirths and neonatal deaths: where, why, and what can be done? Int J Gynecol Obstet. 2009 Oct;107 Suppl 1.
3. Bhatti A, Kumar P. Systemic effects of perina- tal asphyxia. Indian J Pediatr. 2014 Mar;81(3): 231–3.
4. Edwards AD, Brocklehurst P, Gunn AJ, Hal- liday H, Juszcak E, Levene M, et al. Neuro- logical outcomes at 18 months of age after moderate hypothermia for perinatal hypoxic ischaemic encephalopathy: synthesis and meta-analysis of trial data. BMJ. 2010 Feb;340:c363.
5. Hutton JL, Pharoah PO. Life expectancy in se- vere cerebral palsy. Arch Dis Child. 2006 Mar; 91(3):254.
6. Lee AC, Kozuki N, Blencowe H, Vos T, Bah- halim A, Darmstadt GL, et al. Intrapartum- related neonatal encephalopathy incidence and impairment at regional and global levels for 2010 with trends from 1990. Pediatr Res. 2013 Dec;74(Suppl 1):50–72.
7. Jacobs SE, Berg M, Hunt R, Tarnow-Mordi WO, Inder TE, Davis GP. Cooling for new- borns with hypoxic ischaemic encephalopa- thy. Cochrane Database Syst Rev. 2013 Jan 31; 2013(1):CD003311.
8. Bach AM, Fang AY, Bonifacio S, Rogers EE, Scheffler A, Partridge JC, et al. Early magnetic resonance imaging predicts 30-month out- comes after therapeutic hypothermia for neo- natal encephalopathy. J Pediatr. 2021 Nov; 238:94–101.e1.
9. Krishnan V, Kumar V, Shankaran S, Thayyil S. Rise and fall of therapeutic hypothermia in low-resource settings: lessons from the HE- LIX trial. Indian J Pediatr. 2021 Jul. Epub ahead of print.
10. Thayyil S, Pant S, Montaldo P, Shukla D, Oliveira V, Ivan P, et al. Hypothermia for moderate or severe neonatal encephalopathy in low-income and middle-income countries (HELiX): a randomised controlled trial in India, Sri Lanka, and Bangladesh. Lancet Glob Health. 2021 Sep;9(9):e1273–85.
11. Sarnat HB, Sarnat MS. Neonatal encephalop- athy following fetal distress. A clinical and electroencephalographic study. Arch Neurol. 1976 Oct;33(10):696–705.
12. Ahearn CE, Boylan GB, Murray DM. Short and long term prognosis in perinatal asphyxia: an update. World J Clin Pediatr. 2016 Feb; 5(1):67–74.
13. Mota-Rojas D, Villanueva-Garcia D, Solima- no A, Muns R, Ibarra-Ríos D, Mata-Reyes A. Pathophysiology of perinatal asphyxia in hu- mans and animal models. Biomedicine. 2022 Feb;10(2):347.
14. Mengenthaler P, Meisel A. Animal models: value and translational potency. Principles of trans- lational science in medicine. Elsevier; 2021. p. 95–103.
15. Totorou K, Sisa C, Iqbal A, Dhillon K, Hris- tova M. Current therapies for neonatal hy- poxic-ischaemic and infection-sensitised hypoxic-ischaemic brain damage. Front Synap- tical Neurosci. 2021 Aug;13:709301.
16. Derrick M, Drobyshevsky A, Ji X, Tan S. A model of cerebral palsy from fetal hypoxia-ischemia. Stroke. 2007 Feb;38(2):731.
17. Aridas JDS, Yawno T, Sutherland AE, Nitsos I, Ditchfield M, Wong FY, et al. Detecting brain injury in neonatal hypoxic-ischaemic en- cephalopathy: closing the gap between experi- mental and clinical research. Exp Neurol. 2014 Nov;261:281–90.
18. Polglase GR, Schmölzer GM, Roberts CT, Blank DA, Badurdeen S, Crossley KJ, et al. Cardiopulmonary resuscitation of asyto- bolic newborn lambs prior to umbilical cord clamping: the timing of cord clamping mat- ters. Pediatr Res. 2020;110:02.
19. Aridas JDS, Yawno T, Sutherland AE, Nitsos I, Ditchfield M, Wong FY, et al. Systemic and transdermal melatonin administration pre- vents neuropathology in response to perinatal asphyxia in newborn lambs. J Perinatol. 2018 May;46(4):e12479.
20. Yawno T, Castillo-Melendez M, Jenkins K, Wallace EM, Walker DW, Miller SL. Mecha- nisms of melatonin-induced protection in the brain of late gestation fetal sheep in response to hypoxia. Dev Neurosci. 2012;34(6):543.
21. Gunn AJ, Gunn TR, de Haan HH, Williams CE, Gluckman PD. Dramatic neuronal rescue with prolonged selective head cooling after ischemia in fetal lambs. J Clin Invest. 1997 Jan;99(2):248–56.
22. Johnson JJ, Sudheimer KD, Davis KK, Kerndt GM, Winn BM. The sheep brain atlas. East Lansing, MI: Michigan State University; 2009. Available from: https://brains.anatomy.msu.edu/brains/sheep/index.html.
23. Björkman ST, Foster KA, O’driscoll SM, Healy GN, Lingwood BE, Burke C, et al. Hy- poxic/ischemic models in newborn piglet: comparison of constant FiO2 versus variable FiO2 delivery. Brain Res. 2006 Jul;1100(1): 110–7.
24. Dwyer CM, Lawrence AB, Brown HE, Simm G. Effect of ewe and lamb genotype on gestation length, lambing ease and neonatal behaviour of lambs. Reprod Fertil Dev. 1996;8(8):1123.
25. Castillo-Melendez M, Babarumani AA, Ca- balag C, Yawno T, Wittjaksono A, Miller SL, et al. Experimental modelling of the conse- quences of brief late gestation asphyxia on newborn lamb behaviour and brain structure. PLoS One. 2013;8(11)e77377.
