



PLURIPOTENT STEM CELLS

Window of opportunity for human amnion epithelial stem cells to attenuate astrogliosis after umbilical cord occlusion in preterm fetal sheep

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Funding information

Auckland Medical Research Foundation, Grant/Award Number: 1211003; Health Research Council of New Zealand, Grant/Award Numbers: 16/003, 17/601, 12/613

Abstract

There is increasing evidence that administration of many types of stem cells, including human amnion epithelial cells (hAECs), can reduce hypoxic-ischemic injury, including in the perinatal brain. However, the therapeutic window for single dose treatment is not known. We compared the effects of early and delayed intracerebroventricular administration of hAECs in fetal sheep at 0.7 gestation on brain injury induced by 25 minutes of complete umbilical cord occlusion (UCO) or sham occlusion. Fetuses received either 1×10^6 hAECs or vehicle alone, as an infusion over 1 hour, either 2 or 24 hours after UCO. Fetuses were killed for brain histology at 7 days post-UCO. hAEC infusion at both 2 and 24 hours had dramatic anti-inflammatory and anti-gliotic effects, including significantly attenuating the increase in microglia after UCO in the white and gray matter and the number of astrocytes in the white matter. Both protocols partially improved myelination, but had no effect on total or immature/mature numbers of oligodendrocytes. Neuronal survival in the hippocampus was increased by hAEC infusion at either 2 or 24 hours, whereas only hAECs at 24 hours were associated with improved neuronal survival in the striatum and thalamus. Neither protocol improved recovery of electroencephalographic (EEG) power. These data suggest that a single infusion of hAECs is anti-inflammatory, anti-gliotic, and neuroprotective in preterm fetal sheep when given up to 24 hours after hypoxia-ischemia, but was associated with limited white matter protection after 7 days recovery and no improvement in the recovery of EEG power.

KEYWORDS

asphyxia, inflammation, neuroprotection, preterm birth, stem cells

1 | INTRODUCTION

Children born preterm continue to have high risk of neurodevelopmental impairment.¹ In modern cohorts, the pathological

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substrate of this risk is primarily related to local astrogliosis and microglial infiltration leading to maturational arrest of white matter and reduced regional brain volumes.² The mechanisms mediating brain injury and impaired brain development are multifactorial, but perinatal asphyxia and inflammation are significant factors.³ Currently, except for maternal magnesium therapy for preterm birth,⁴ there are no established treatments available to improve outcomes in high risk preterm infants.

The challenges in developing treatments for preterm infants include the uncertain timing of injury and multiple exposures to adverse events. Injury evolves slowly after the resolution of an adverse event and the particular phase of injury may dramatically alter the efficacy of the treatment. For example, both experimental and clinical data show that therapeutic hypothermia, currently used to treat hypoxic-ischemic encephalopathy (HIE) in term and near-term infants, is effective when begun in the early hours after an insult, but loses efficacy over time as mitochondrial function becomes impaired.^{5,6} However, currently we lack biomarkers to determine when an injurious event has occurred during pregnancy and injury is commonly already present by the time of birth.^{7,8} Equally, babies may be exposed to multiple adverse events before, during, and after birth, changing the evolution of injury and the mechanisms mediating injury, making targeted treatment difficult to develop.⁹

Treatments that are effective at modulating multiple mechanisms of injury, and that can augment endogenous neuroprotection processes could in principle address some of these issues. Multipotential ("stem") cell therapy may be one such option.¹⁰⁻¹³ The potential neuroprotective efficacy of stem cell treatments appear to be mediated by multiple protective effects including the ability to promote proliferation, growth, and differentiation through release of trophic factors and chemokines to support host cell survival and development, and their ability to modulate the inflammatory responses after hypoxia-ischemia.¹¹⁻¹³ A variety of different stem cells are currently being assessed clinically and pre-clinically, including human amniotic epithelial cells (hAECs).^{10,13,14} These cells have many benefits over other types of stem-cell treatments, including the fact that they can be readily harvested from amniotic membranes discarded at birth and do not require invasive extraction, making them readily available for rapid treatment in the early postnatal phase.¹⁵ They are pluripotent, are capable of differentiating into cell types of all three germ layers, are nontumorigenic, nonimmunogenic, and have significant immunomodulatory properties, making them ideal as a generic therapy.^{11,16}

In adult animals, hAECs have been shown to reduce cerebral infarct size and improve behavioral functionality in memory and motor coordination after an ischemic stroke,¹⁷ to reduce brain swelling and improve motor function after intracerebral hemorrhage and to reduce inflammation in experimental autoimmune encephalitis.¹⁸ In near-term fetal sheep, hAECs have been shown to ameliorate white and gray matter injury induced by the inflammatory mediator lipopolysaccharide,¹⁹ in association with reduced microglial activation, suggesting a reduction in inflammation. Similarly, hAECs reduced ventilation-induced inflammatory white matter injury in newborn lambs,²⁰ and lung injury related to ventilation,²¹ and hyperoxia.²² We have recently shown that delayed

Significance statement

Preterm infants have high rates of disability, which is linked to secondary neural inflammation and astrogliosis leading to white matter maturational failure. It is a formidable challenge to start potential treatments in the critical interval after injury. This article reports that, in preterm fetal sheep, amnion epithelial cells given either 2 or 24 hours after asphyxia, similarly attenuate neuronal loss and microglial and astrocyte upregulation and partially improve myelination but did not improve recovery in brain activity. This suggests that cell treatments have a broad therapeutic window and so may have strategic value to help alleviate maturational failure.

intranasal infusions of hAECs, given at 1, 3, and 10 days after 25 minutes of complete umbilical cord occlusion, was associated with improved brain weight, improved oligodendrocyte maturation and myelination, and reduced microglia and astrocyte number after 21 days recovery in the preterm fetal sheep.²³ Furthermore, in a similar paradigm in preterm fetal sheep, intravenous infusion of umbilical cord blood derived mesenchymal stem/stromal cells at 12 hours but not 5 days after umbilical cord occlusion reduced white matter injury and cerebral inflammation.²⁴

In the present study, we sought to better understand the relationship between the timing of administration of hAECs and the phases of evolving injury after hypoxia-ischemia and their effects on white and gray matter injury, and cerebral inflammation. hAECs were given as a single intracerebroventricular injection to preterm fetal sheep at 0.7 gestation after profound, reversible asphyxia in utero, either in the early latent phase (2 hours) or at 24 hours, after the onset of secondary energy failure.²⁵ At this age, the fetal sheep brain development is broadly equivalent to that at 28 to 32 weeks in humans.²⁶

2 | MATERIALS AND METHODS

2.1 | Ethical approval

All procedures were approved by the Animal Ethics Committee of the University of Auckland and in accordance with the Animal Welfare Act (1999) of New Zealand and reported according to the ARRIVE guidelines (Animal Research: Reporting in vivo Experiments). Human amnion epithelial stem cells were collected from the placentae of women with uncomplicated pregnancies undergoing elective cesarean section at term. Tissue collection was performed with approval from the Monash Health Human Research Ethics Committee, Monash, Melbourne, Australia. Women gave written, informed consent for the collection of their placentae, and all procedures conformed to the standards set by the Declaration of Helsinki.

2.2 | Surgical procedures

Food, but not water, was withdrawn 12 to 18 hours before surgery to reduce vomiting during surgery. Thirty-two ewes were given long acting oxytetracycline (20 mg/kg, Phoenix Pharm Distributors Ltd, Auckland, New Zealand) intramuscularly 30 minutes before surgery for prophylaxis. Anesthesia was induced by i.v. injection of propofol (5 mg/kg, AstraZeneca PLC, London, UK) and general anesthesia was maintained using 2% to 3% isoflurane in oxygen. The depth of anesthesia (maternal muscle tone and reflexes, heart rate, and respiration) was constantly monitored by trained anesthetic staff. Ewes received a slow infusion of isotonic saline (approximately 250 mL/hour) to maintain fluid balance.

Fetuses were 99 to 100 days of gestation at the time of surgery (term ~147 days gestation). Following maternal laparotomy, a midline abdominal incision was made to expose the uterus, and the fetus was partially exteriorized for instrumentation. Polyvinyl catheters (SteriHealth, Dandenong South, Victoria, Australia) were placed in the left fetal femoral artery to measure blood pressure and the right brachial artery for pre-ductal blood sampling. An additional catheter was placed into the amniotic sac for the measurement of amniotic fluid pressure. An ultrasound flow probe (size 3S, Transonic Systems, Ithaca, New York) was placed around the left carotid artery to measure carotid artery blood flow, as an index of cerebral blood flow.²⁷ A pair of electrodes was placed across the fetal chest to measure the fetal electrocardiogram (ECG, Cooner Wire, Chatsworth, California) from which fetal heart rate (FHR) was derived. Two pairs of electrodes (Cooner Wire) were placed over the parietal dura bilaterally, 5 and 10 mm anterior to bregma and 10 mm lateral to the sagittal suture to record brain wave activity. A reference electrode was sewn over the occiput.

A 14 mm, 22-gauge cannula attached to a catheter was placed in the right lateral ventricle, 6 mm anterior, and 4 mm lateral to bregma for intracerebroventricular administration of hAECs or vehicle. An inflatable silicone occluder (In Vivo Metric, Healdsburg, California) was placed around the umbilical cord to facilitate umbilical cord occlusion after surgery. Prior to closure of the uterus, ~300 mL of warm sterile isotonic saline was added to the uterus to replace lost amniotic fluid, along with the antibiotic gentamicin sulfate (80 mg, Pfizer Pty Ltd, Perth, Australia). For analgesia, the maternal midline skin incision was infiltrated with 10 mL 0.5% bupivacaine plus adrenaline (Marcain, AstraZeneca Ltd, Auckland, New Zealand). All fetal leads were exteriorized through the maternal flank, and a maternal long saphenous vein was catheterized for postoperative care and euthanasia.

2.3 | Postsurgical recovery

After surgery, ewes were housed in separate metabolic cages with companion animals in a temperature-controlled room ($16 \pm 1^\circ\text{C}$, humidity $50\% \pm 10\%$) with a 12-hour light/dark cycle (light 0600 to 1800 hours) and ad libitum access to water and food. Postsurgical care, animal monitoring, and animal laboratory management were

undertaken in accordance with animal ethics approval and the animal and facility standard operating procedures approved by the Animal Ethics Committee of The University of Auckland.

Ewes and their fetuses were allowed to recover for 4 to 5 days before experiments commenced. Ewes received i.v. antibiotics daily for 4 days (600 mg benzylpenicillin sodium; Novartis Ltd, Auckland, New Zealand, and 80 mg gentamicin sulfate). Fetal catheters were maintained patent by continuous infusion of heparinized saline (20 U/mL at 0.2 mL/h) and the maternal catheter was flushed daily with heparinized saline. Physiological parameters were recorded continuously from 24 hours before until the end of experiment 7 days after umbilical cord occlusion. At the end of the experiment, the ewes and their fetuses were killed for tissue collection by an intravenous overdose to the ewe of sodium pentobarbitone (9 g; Pentobarb 300, Chemstock International, Christchurch, New Zealand).

2.4 | Data recording

Fetal mean arterial blood pressure (MAP), FHR, carotid blood flow (CaBF), and electroencephalographic activity (EEG) signals were recorded continuously from 24 hours before until 7 days after umbilical cord occlusion. All signals were digitized at a sampling rate of 4096 Hz and the signal decimated to lower rates for data analysis. Data were stored on computer and processed using custom acquisition software (Labview for Window, National Instruments, Austin, Texas). Fetal blood pressure was recorded using Novatrans II, MX860 pressure transducers (Medex Inc, Hilliard, Ohio) and corrected for maternal movement by subtraction of amniotic fluid pressure.

Signals were processed as previously described.²⁸ The blood pressure signal was collected at 64 Hz and low-pass filtered at 30 Hz. The raw ECG signal was analogue filtered with a first order high-pass filter set at 0.05 Hz and an eighth order low-pass Bessel filter set at 100 Hz. Continuous CaBF was measured using a two-channel Transonic T-206 Flowmeter and data were 10 Hz low-pass filtered with a second order Butterworth filter. The raw ECG signal was analogue filtered with a first order high-pass filter set at 0.05 Hz and an eighth order low-pass Bessel filter set at 100 Hz. The EEG signal was processed with a first-order high-pass filter at 1.6 Hz and a sixth-order Butterworth low-pass filter with the cutoff frequency at 256 Hz, and then digitally stored at a sampling rate of 512 Hz. EEG power was derived from the power spectrum between 1 and 20 Hz and log transformed (decibels [dB], $10 \times \log[\text{power}]$) as this transformation gives a better approximation of the normal distribution. Spectral edge was defined as the frequency below which 90% of total EEG power was present, as a measure of the relative frequency of the EEG.²⁹

2.5 | Preparation of hAECs

hAECs were isolated from term human placentae donated by healthy volunteers who underwent elective cesarean section

delivery as described previously.³⁰ Briefly, the amnion was manually separated from the chorion, and blood removed by repeated washing in Hanks' Balanced Salt Solution. The hAECs were enzymatically removed by two 1-hour digests using 0.25% (v/v) Trypsin-EDTA (Thermo Fisher Scientific) and inactivation was performed by the addition of 1% (v/v) Newborn Calf Serum (Thermo Fisher Scientific). The hAECs were collected by centrifugation for 10 minutes at 500g and resuspended for cell counts and viability testing. As previously characterized, hAECs are epithelial cell adhesion molecule positive but negative for CD31, CD45, CD90, and CD105. Cells were cryopreserved in FBS containing 10% (v/v) dimethyl sulfoxide (DMSO) at 5×10^6 cells/mL. Frozen cells were thawed, washed, counted, and placed in culture 24 hours before administration.³⁰ Prior to administration, frozen cells were nearly thawed in a 37°C water bath and washed thrice in DMEM/F12 to remove DMSO. Cells were then resuspended in 10 mL DMEM/F12 for cell counting and viability testing using trypan blue exclusion. The cells were then resuspended in the appropriate amount of DMEM/F12 to seed ($\sim 10 \times 10^6$ cells per T175 flask). Cells were cultured in DMEM/F12 with 10% FBS, 1% Antibiotic-Antimycotic (Invitrogen, Carlsbad, California) and 1% L-Glutamine (Sigma-Aldrich, St Louis, Missouri) for 24 hours before administration. Cells were harvested for administration by incubation with 2.5% trypsin/phosphate buffered saline (PBS) for 10 minutes at 37°C then further diluted in DMEM and spun down at 450g for 5 minutes. Cells were then counted and the viability assessed as above. Average viability before final suspension was $85.25\% \pm 1.06\%$ in the 2 hour and $81.71\% \pm 2.9\%$ in the 24 hour groups. For infusion, 1.5 million cells were suspended in 1 mL of sterile PBS.

2.6 | Experimental protocols

Fetal asphyxia was induced at 105 ± 1 days of gestation. Fetuses were randomly assigned to the following groups: sham-sham group ($n = 8$), asphyxia-vehicle group ($n = 8$), asphyxia-2 hour hAEC group ($n = 8$), and asphyxia-24 hour hAEC group ($n = 8$). Asphyxia was induced by complete umbilical cord occlusion for 25 minutes. Successful occlusion was determined by heart rate and blood pressure changes, and blood samples taken for pH and blood gas analysis. All occlusions were started in the morning between 9 and 9.30 AM. Two hours or 24 hours after occlusion, artificial cerebrospinal fluid³¹ or hAECs were infused into the lateral ventricle over 20 minutes (0.05 mL/hour) and the catheter dead-space (~ 0.7 mL) cleared over a further 20 minutes with an infusion of artificial cerebrospinal fluid.

Fetal arterial blood samples were collected at 1 hour before, 5 and 17 minutes during, and 1, 2, 4, and 6 hours after occlusion. Daily samples were then taken between 8 and 9 AM. Fetal pre-ductal pH, PaCO₂, PaO₂ (Ciba-Corning Diagnostics 845 blood gas analyzer and co-oximeter, Massachusetts), and glucose and lactate (YSI model 2300, Yellow Springs, Ohio) measurements were made. The fetuses were monitored for 7 days after occlusion.

2.7 | Fetal cytokine measurements

Additional blood samples were collected 1 hour before occlusion, and at 3, 6, 24 hours after occlusion and then daily for measurement of cytokine levels using in-house enzyme linked immunosorbent assay.^{32,33} Ovine-specific antibodies were used to measure fetal plasma for tumor necrosis factor (TNF), interleukin (IL)-6 (Epitope technologies, Melbourne, Australia), whereas bovine antibodies were used to measure IL-10 (AbD Serotec, MorphoSys, Kidlington, UK). Standards were ovine recombinant TNF (range: 0-10 ng/mL, detection sensitivity 0.354 ng/mL) and IL-6 (range: 0-5 ng/mL, detection sensitivity 0.097 ng/mL; Protein Express, Cincinnati, Ohio), and recombinant bovine IL-10 (range: 0-11 biological units [BU]/mL, detection sensitivity 0.086 BU/mL; supplied by Prof. G Entrican, Moredun Research Institute, Midlothian, Scotland). Internal quality controls were included in each assay and cytokine concentrations were within the detection limit in all samples.

2.8 | Histology

Fetal brains were perfusion fixed with 10% formalin before being embedded in paraffin, 10 μ m sections were cut and assessed. Slides were coded for blinded analysis of the data. Slides were dewaxed and rehydrated. Citrate antigen retrieval was performed for all antigens except for Olig-2, followed by endogenous peroxidase quenching. Blocking was performed with 3% normal horse serum in PBS for Iba-1 or 3% normal goat serum in PBS for all other stains for 1 hour at room temperature. Sections were labelled with monoclonal primary antibodies: 1:200 concentration anti-ionized calcium binding adaptor molecule 1 (Iba-1, a marker for activated microglia, Abcam, Cambridge, Massachusetts), 1:400 rabbit anti-oligodendrocyte transcription factor 2 (Olig2, a marker for all stages of the oligodendrocyte lineage, Merck-Millipore Corporation, Billerica, Massachusetts), 1:200 mouse anti-2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNPase, a marker of immature and mature oligodendrocytes, Merck-Millipore), 1:200 rabbit anti-myelin basic protein (MBP, Merck-Millipore), 1:200 rabbit anti-gial fibrillary acidic protein (GFAP, Abcam), or 1:200 mouse anti-STEM121 (a human stem cell marker, Stem Cells Inc, Newark, California), 1:400 anti-neuronal nuclear antigen (NeuN, a marker of mature neurons, Merck-Millipore), and anti-Ki-67 1:200 (a marker of proliferation, Dako, Carpinteria, California). STEM121 was used to identify hAECs.

Sections labeled with these primary antibodies were incubated overnight at 4°C, then washed and incubated with secondary biotinylated monoclonal anti-rabbit 1:200 or anti-mouse 1:200 IgG antibodies (Vector Laboratories, Burlingame, California) for 3 hours at room temperature. Slides were incubated in ExtrAvidin 1:200 (Sigma Aldrich Pty. Ltd) for 2 hours at room temperature and then reacted with DAB (Sigma Aldrich Pty. Ltd).

Photomicrographs were acquired by light microscopy at $\times 20$ or $\times 40$ magnification on a Nikon 80i microscope and NIS Elements Br 4.0 software (Nikon Instruments Inc, Melville, New York). Cells were

counted from three fields in the white matter (first intragyral [IGWM1], second intragyral [IGWM2], and periventricular [PVWM]), three areas in the cortex (first and second parasagittal gyrus [1PSG and 2PSG, respectively] and lateral cortex [LC]), three areas in the hippocampus (cornu ammonis [CA] CA1/2, CA3 and dentate gyrus [DG]) as well as seven fields in the striatum (four in the caudate nucleus, three in the putamen) and three fields in the thalamus. Cells were counted on two slides in both hemispheres for each animal.

2.9 | Data analysis

Off-line physiological data analysis was performed using customized programs (Labview for Windows). Baseline data represent 1 hour average prior to umbilical cord occlusion. Continuous raw EEG traces of all fetuses were assessed for the appearance of large amplitude stereotypic evolving seizures. Seizures were defined as EEG events consisting of rhythmic repetitive waves occurring for at least 10 seconds, which vary in frequency or amplitude as the seizure progresses.³⁴ Seizure number, duration, and peak amplitude were determined. Mean interictal EEG amplitude was manually determined during the 35th hour as the percentage change from baseline. Carotid vascular resistance (CaVR) was calculated using the formula (MAP/CaBF [mmHg/minute/mL]). The correlation coefficient was calculated for EEG power vs CaBF, or NeuN positive neurons in the CA1/2 or CA3.

2.10 | Statistics

For analysis of the physiological data, hourly averages of minute data from 24 hours of baseline and 7 days of experiment were analyzed and between group comparisons were done with repeated measures ANOVA. When a statistically significant effect was found, post-hoc LSD pairwise comparisons were used between groups (SPSS v22, SPSS Inc, Chicago, Illinois). For analysis of the histology data, repeated measures ANOVA was performed across areas, and where significance was found, followed by LSD post hoc comparisons. As cytokine data were not normally distributed, data were log transformed and differences between groups were assessed by repeated measures ANOVA and changes from baseline were assessed using Dunnett's test. Statistical significance was accepted at $P < .05$. Data are mean \pm standard error of the mean (SEM) except for cytokine data, which are presented as median \pm interquartile range.

3 | RESULTS

3.1 | Biochemistry

There were four males and four females in the sham control group, four males and four females in the asphyxia-vehicle group, five

females and three males in the 2-hour treatment group and one female and seven males in the 24-hour treatment group. There were no differences in baseline physiological recordings between groups. Cord occlusion was associated with significant mixed acidosis, hypercapnia, and hypoxia compared to the sham-sham group (Table 1), but there were no differences between the asphyxia groups. The time course for resolution of pH, blood gases, and glucose and lactate was similar between asphyxia groups. There was no significant difference between the asphyxia-vehicle and asphyxia-hAEC groups for IL-6, IL-10, or TNF- α (Supplemental Figure 1), and no significant change over time.

3.2 | Hemodynamic responses

Umbilical cord occlusion (UCO) was characterized by profound bradycardia, hypotension and carotid hypoperfusion, which was not significantly different between asphyxia groups. During the last minute of cord occlusion, MAP for the asphyxia-vehicle (13.7 ± 1.4 mmHg), asphyxia-2 hour hAEC (12.1 ± 1.2 mmHg) and asphyxia-24 hour hAEC (12.8 ± 2.0 mmHg) groups were all significantly lower compared to the sham-sham group (35 ± 1.1 mmHg, $P < .05$). FHR was significantly reduced for the asphyxia-vehicle (61.8 ± 2.3 bpm), asphyxia-2 hour hAEC (59.3 ± 3.5 bpm) and asphyxia-24 hour hAEC (62.5 ± 3.8 bpm) groups compared to the sham-sham group (191 ± 2.3 bpm, $P < .05$). Finally, CaBF in the sham-sham group was (29.1 ± 3.0 mL/minutes) during the last minute of UCO, and was significantly reduced in the asphyxia-vehicle (10.6 ± 2.0 mL/minute), asphyxia-2 hour hAEC (10.1 ± 2.1 mL/minute) and asphyxia-24 hour hAEC groups (10.1 ± 2.1 mL/minute, $P < .05$).

UCO was associated with transient tachycardia compared to the sham-sham group during the first 4 hours, with peak heart rate at 3 hours (asphyxia-vehicle 221 ± 11 bpm, 2-hour treatment group 229 ± 11 bpm and 24-hour treatment group 212 ± 12 bpm compared to the sham-sham group 190 ± 5 bpm, $P < .05$; Figure 1A). Thereafter, there were no differences between groups. A diurnal rhythm in FHR was reestablished in the asphyxia groups from around 24 hours postocclusion.

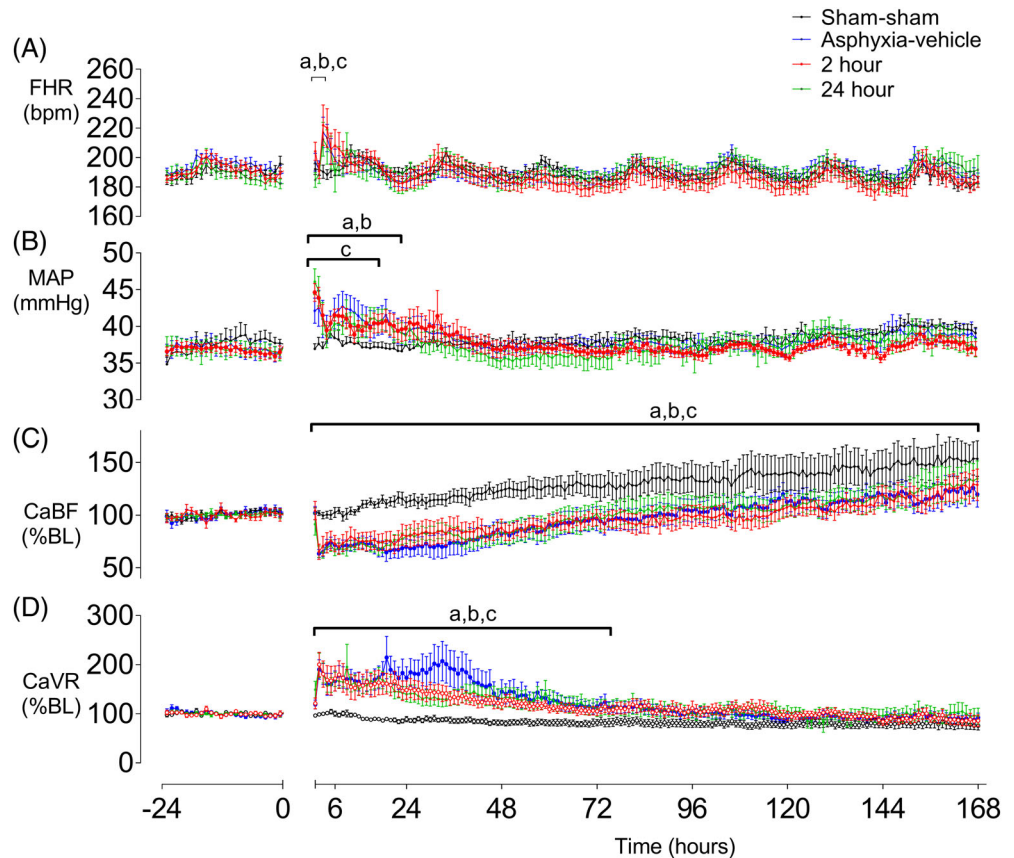
During recovery, MAP was transiently elevated during the first 29 hours in the asphyxia-vehicle and 2 hour hAEC groups and for the first 24 hours in the 24 hour hAEC group, with pressure peaking around 1 hour postocclusion; at 42.4 ± 1.6 mmHg in the asphyxia-vehicle group, 42.4 ± 1.6 mmHg in the 2 hour hAEC group and 46.1 ± 1.7 mmHg in the 24 hour hAEC group compared to 36.9 ± 0.7 mmHg in the sham-sham group ($P < .05$; Figure 1B). There was no significant difference between asphyxia groups, or between asphyxia groups and the sham-sham group for the remainder of the experiment.

UCO was associated with a significant reduction in CaBF. Although CaBF partially recovery after the end of occlusion, it remained lower than sham-sham values until the end of the experiment ($P < .05$; Figure 1C). This was accompanied by a significant increase in CaVR from 1 to 77 hours after UCO in the asphyxia groups

TABLE 1 Fetal arterial pH, blood gas and glucose lactate measurements after asphyxia for sham-sham, asphyxia-vehicle (Asphyxia), and 2 and 24 hour human amniotic epithelial cell (hAEC) treatment (2 hour hAEC, 24 hour hAEC) groups. Measurements were made 1 hour before, at 17 minutes during, 1, 2, 4, and 6 hours immediately after asphyxia and then selected daily time points are presented for the remainder of the study. * $P < .05$ vs the sham-sham group. Data are means \pm SEM

	Baseline	17 minutes	1 hour	2 hours	4 hours	6 hours	D1	D3	D5	D7
pH	Sham-sham	7.38 \pm 0.0	7.37 \pm 0.0	7.37 \pm 0.0	7.37 \pm 0.0	7.36 \pm 0.0	7.37 \pm 0.0	7.36 \pm 0.0	7.37 \pm 0.0	7.37 \pm 0.0
	Asphyxia	7.38 \pm 0.0	6.83 \pm 0.0*	7.30 \pm 0.0	7.34 \pm 0.0	7.41 \pm 0.0	7.37 \pm 0.0	7.38 \pm 0.0	7.39 \pm 0.0	7.38 \pm 0.0
PaCO ₂ (mmHg)	2 hours hAEC	7.36 \pm 0.0	6.83 \pm 0.0*	7.27 \pm 0.0	7.32 \pm 0.0	7.41 \pm 0.0	7.37 \pm 0.0	7.37 \pm 0.0	7.38 \pm 0.0	7.37 \pm 0.0
	24 hours hAEC	7.38 \pm 0.0	6.86 \pm 0.0*	7.27 \pm 0.0	7.34 \pm 0.0	7.41 \pm 0.0	7.38 \pm 0.0	7.37 \pm 0.0	7.37 \pm 0.0	7.37 \pm 0.0
PaO ₂ (mmHg)	Sham-sham	46.3 \pm 1.2	50.0 \pm 1.4	47.1 \pm 1.2	50.1 \pm 1.5	46.5 \pm 1.1	50.0 \pm 1.3	48.3 \pm 1.2	49.1 \pm 1.8	50.7 \pm 1.2
	Asphyxia	50.6 \pm 1.2	147.5 \pm 3.7*	43.8 \pm 0.9	45.3 \pm 1.3	44.6 \pm 0.9	48.0 \pm 0.6	47.9 \pm 0.8	48.3 \pm 1.1	48.9 \pm 1.4
Glu (mmol/L)	2 hours hAEC	51.6 \pm 1.3	150.8 \pm 8.3*	44.2 \pm 2.4	43.6 \pm 1.0*	45.4 \pm 0.6	46.7 \pm 1.3	48.1 \pm 1.1	49.2 \pm 1.7	48.0 \pm 1.2
	24 hours hAEC	52.6 \pm 0.7	134.1 \pm 5.4*	42.6 \pm 0.9*	45 \pm 1.7*	43.7 \pm 0.9*	47.9 \pm 1.2	48.3 \pm 1.3	47.9 \pm 1.6	50.6 \pm 2.5
Lac (mmol/L)	Sham-sham	25.3 \pm 1.0	25.0 \pm 1.5	26.0 \pm 1.1	26.0 \pm 1.7	24.7 \pm 1.2	26.0 \pm 1.2	25.1 \pm 1.2	24.5 \pm 1.3	25.3 \pm 1.1
	Asphyxia	24.8 \pm 1.0	8.0 \pm 1.0*	28.9 \pm 1.5*	24.9 \pm 1.6	24.8 \pm 1.6	25.2 \pm 1.6	27.9 \pm 1.1	25.9 \pm 1.6	26.3 \pm 1.8
pH	2 hours hAEC	23.7 \pm 1.2	9.7 \pm 0.5*	30.7 \pm 1.8*	27.7 \pm 1.4	26.3 \pm 1.1	26.1 \pm 1.0	29.0 \pm 1.1	26.2 \pm 1.1	26.5 \pm 1.4
	24 hours hAEC	23.0 \pm 1.0	7.0 \pm 0.6*	28.9 \pm 2.0*	24.1 \pm 0.8	25.5 \pm 1.9	25.5 \pm 1.3	26.7 \pm 1.9	27.2 \pm 1.7	22.6 \pm 2.5
pH	Sham-sham	1.0 \pm 0.1	1.1 \pm 0.0	1.1 \pm 0.0	1.2 \pm 0.1	1.1 \pm 0.0	1.0 \pm 0.0	1.2 \pm 0.1	1.0 \pm 0.0	1.0 \pm 0.0
	Asphyxia	1.0 \pm 0.0	0.7 \pm 0.1*	1.3 \pm 0.1*	1.3 \pm 0.1	1.3 \pm 0.1*	1.5 \pm 0.1*	1.2 \pm 0.1	1.0 \pm 0.1	1.1 \pm 0.1
pH	2 hours hAEC	1.1 \pm 0.0	0.8 \pm 0.1*	1.4 \pm 0.1*	1.4 \pm 0.0*	1.4 \pm 0.0*	1.6 \pm 0.1*	1.4 \pm 0.2*	1.1 \pm 0.0*	1.1 \pm 0.0
	24 hours hAEC	1.2 \pm 0.2	0.7 \pm 0.1*	1.5 \pm 0.2*	1.4 \pm 0.1	1.2 \pm 0.1	1.6 \pm 0.3*	1.6 \pm 0.1*	1.0 \pm 0.1	1.1 \pm 0.1
pH	Sham-sham	0.9 \pm 0.0	0.8 \pm 0.1	0.8 \pm 0.1	0.9 \pm 0.1	0.8 \pm 0.0	0.9 \pm 0.0	0.8 \pm 0.1	0.8 \pm 0.1	0.8 \pm 0.1
	Asphyxia	0.9 \pm 0.1	6.7 \pm 0.4*	4.9 \pm 0.2*	4.2 \pm 0.9*	2.3 \pm 0.5*	2.0 \pm 0.3*	1.6 \pm 0.4*	0.9 \pm 0.1	0.7 \pm 0.1
pH	2 hours hAEC	0.8 \pm 0.0	6.3 \pm 0.3*	4.6 \pm 0.3*	3.9 \pm 0.4*	2.2 \pm 0.2*	1.9 \pm 0.1*	1.2 \pm 0.2	0.8 \pm 0.1	0.8 \pm 0.1
	24 hours hAEC	1.0 \pm 0.1	6.6 \pm 0.8*	5.6 \pm 0.8*	4.8 \pm 1.2*	3.4 \pm 0.9*	3.3 \pm 0.6*	1.3 \pm 0.2	1.0 \pm 0.1	0.8 \pm 0.1

FIGURE 1 Fetal heart rate (FHR, A), mean arterial pressure (MAP, B), carotid blood flow (CaBF, C), and carotid vascular resistance (CaVR, D) during the 24 hours before asphyxia (asphyxia period not shown) and for 168 hours after asphyxia in sham-sham, asphyxia-vehicle, 2 hour human amniotic epithelial cell (hAEC) and 24 hour hAEC groups. (a $P < .05$ asphyxia-vehicle vs sham-sham), (b $P < .05$ 2 hour hAEC vs sham-sham), (c $P < .05$ 24 hour hAEC vs sham-sham). Data are hourly means \pm SEM



compared to the sham-sham group ($P < .05$; Figure 1D). Thereafter, CaVR returned to control group values. There were no significant differences in CaBF or CaVR between UCO groups.

3.3 | EEG power and spectral edge frequency

EEG power was suppressed in all asphyxia groups after umbilical cord occlusion, and remained significantly lower compared to the sham-sham group until the end of the experiment, with no effect of treatment ($P < .05$; Figure 2A). Spectral edge frequency was also suppressed after asphyxia and remained significantly lower than the sham-sham group in the asphyxia 24-hour group until 96 hours and in the asphyxia-vehicle and asphyxia-2 hours group until 144 hours ($P < .05$; Figure 2B).

3.4 | Seizures and interictal EEG activity

No seizures were observed in the sham-sham group. Seizures were observed in all asphyxia groups. There were no differences between the asphyxia-vehicle and the 2 and 24 hour hAEC groups, respectively, for the time to onset of seizures (10.8 ± 3.5 vs 11.0 ± 4.4 and 12.5 ± 3.9 hours), total number of individual seizures (101.6 ± 23.0 vs 103.0 ± 4.4 and 99.8 ± 7.2), duration of individual seizures (76.0 ± 6.0 vs 72.0 ± 7.0 seconds and 72.9

± 8.8 seconds), or peak amplitude of individual seizures ($171.0 \pm 16.0 \mu\text{V}$ vs $168.0 \pm 23.2 \mu\text{V}$ and $160.8 \pm 38.4 \mu\text{V}$). There was a nonsignificant tendency for the total period of seizures to be shorter in the asphyxia-hAEC groups (26.8 ± 5.6 and 28.7 ± 7.2 hours, respectively) than after asphyxia-vehicle (43.1 ± 11.6 hours).

There was a trend to greater suppression of EEG power in the 2 hour treatment group compared to the asphyxia-vehicle group between 30 and 42 hours (2 hour treatment group vs asphyxia-vehicle $P = .078$, 24 hour treatment group vs asphyxia vehicle $P = .987$). The nadir of EEG suppression was at 35 hours postocclusion in the asphyxia-2 hour group. The mean interictal amplitude during the 35th hour was significantly lower in the 2 hour hAEC group ($10.1\% \pm 0.6\%$ of baseline) than the 24 hour group ($16.7\% \pm 0.9\%$, $P = .029$) and the asphyxia-vehicle group ($16.6\% \pm 0.8\%$, $P = .045$). In the 2 hour hAEC group, a subgroup of three animals accounted for the low EEG power. Correlation analysis within the asphyxia-2 hour treatment group, showed no correlation between the average EEG power and average CaBF over 30 to 42 hours postasphyxia ($R^2 = 0.01$), average EEG power and number of NeuN positive neurons in the CA1/2 ($R^2 = 0.07$) or CA3 ($R^2 = 0.06$) of the hippocampus. At 35 hours postasphyxia, the EEG power of the high vs low power 2 hour subgroups was 11.1 ± 3.3 vs 4.0 ± 1.7 dB ($P = .001$), respectively, but CaBF was not significantly different ($84.6\% \pm 8.2\%$ vs $90.4\% \pm 22.5\%$ baseline, $P = .24$).

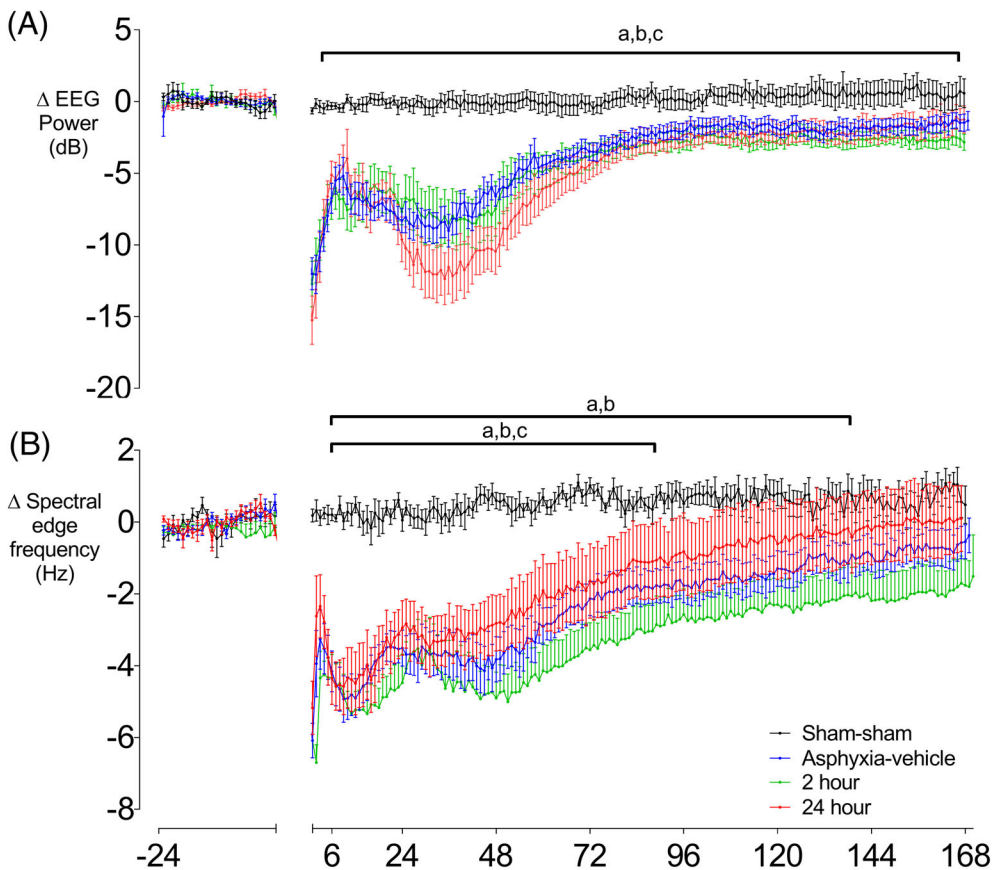


FIGURE 2 Electroencephalographic (EEG) power (A) and spectral edge frequency (B) in the sham-sham, asphyxia-vehicle, 2 hour human amniotic epithelial cell (hAEC) treatment and 24 hour hAEC treatment groups from 24 hours before, until 168 hours after asphyxia. (a $P < .05$ asphyxia-vehicle vs sham-sham), (b $P < .05$ 2 hour vs sham-sham), (c $P < .05$ 24 hour vs sham-sham). Data are hourly means \pm SEM

3.5 | Histology

3.5.1 | White matter injury

Asphyxia was associated an overall reduction in total numbers of oligodendrocytes (Olig-2 positive cells) and of immature/mature (CNPase-positive) oligodendrocytes in all white matter regions compared to the sham-sham group ($P < .05$). There was no significant overall difference between asphyxia groups in any region examined (Figure 3A,B,E). Asphyxia was associated with an overall reduction in MBP-positive cells and area fraction in all regions ($P < .05$; Figure 3C-E). The 2 and 24 hour hAEC groups had lower MBP-positive cell count and area fraction than the sham-sham group ($P < .05$) but the 24 hour hAEC group showed greater numbers of MBP-positive cells than either asphyxia-vehicle or 2 hour hAEC groups ($P < .05$). Moreover, both hAEC groups showed a significantly greater overall MBP area fraction than asphyxia-vehicle ($P < .05$).

STEM-121-positive hAECs were observed in the PVWM at day 7 in both hAEC groups (Supplemental Figure 2).

3.6 | Gray matter injury

Asphyxia-vehicle was associated with an overall reduction in numbers of neurons across the CA1/2, CA3, and dentate gyrus of the hippocampus and the striatum and thalamus compared to

sham-sham ($P < .05$; Figure 4A,C). Numbers of neurons were reduced in the 24 hour hAEC group but not the 2 hour hAEC group across all regions of the hippocampus compared to sham-sham, and both groups had increased numbers of neurons compared to asphyxia-vehicle. There was no significant difference between hAEC groups. There were increased numbers of neurons in the striatum and thalamus in the 24 hour, but not the 2 hour, hAEC group compared to asphyxia-vehicle ($P < .05$; Figure 4B,C). There was no significant difference between the hAEC groups and the sham-sham group.

3.7 | Neuroinflammatory response

There was a marked overall increase in Iba-1-positive microglia across white matter regions in the asphyxia-vehicle group, which was significantly attenuated, to sham-sham levels, in both the 2 and 24 hour hAEC groups ($P < .05$; Figure 5A,E). Similarly, there was an overall increase in Iba-1-positive microglia in the gray matter regions in the asphyxia-vehicle group, which was significantly attenuated to sham-sham levels, in both the 2 and 24 hour hAEC groups ($P < .000$; Figure 5B,E).

Asphyxia was associated with a significant overall increase in both the number of GFAP-positive astrocytes and area fraction of GFAP labelling ($P < .05$; Figure 5C-E). GFAP-positive cell number and area fraction were significantly reduced to sham-sham levels in both the

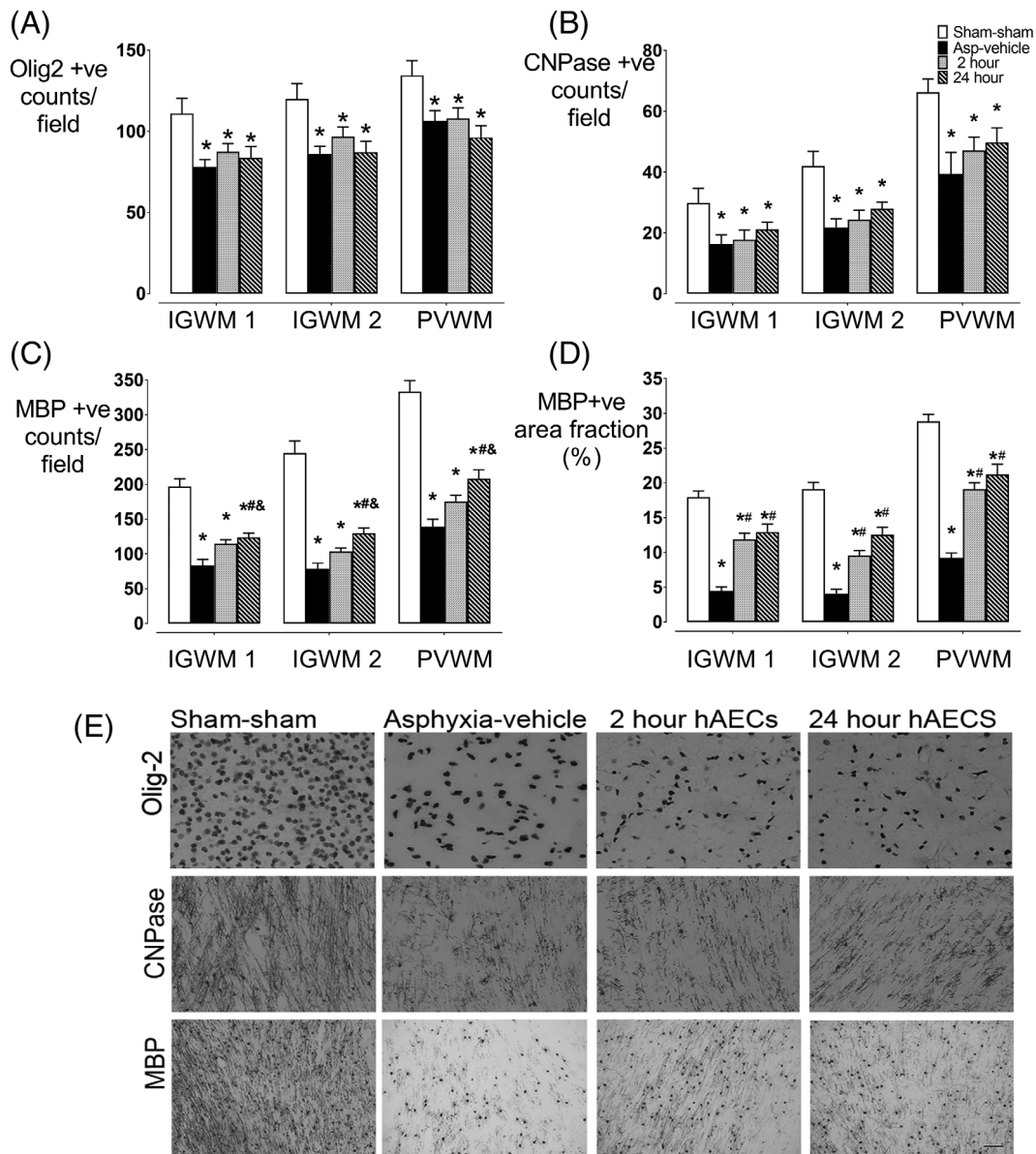


FIGURE 3 Numbers of oligodendrocytes and extent of myelination after asphyxia. Number of oligodendrocyte transcription factor 2 (Olig-2)-positive oligodendrocytes (A), 2',3'-cyclic-nucleotide 3'-phosphodiesterase (CNPase)-positive oligodendrocytes (B), myelin basic protein (MBP)-positive oligodendrocytes (C), and area fraction of MBP expression (D) in the intragryal white matter of the first and second parasagittal gyri (IGWM1 and IGWM2 respectively) and the periventricular white matter (PVWM), 7 days after asphyxia in the preterm fetal sheep. Sham-sham group (white), asphyxia-vehicle group (black), 2 hour treatment group (gray) and 24 hour treatment group (striped). Photomicrographs (E) of Olig-2-positive cells, CNPase-positive oligodendrocytes and MBP in the PVWM. Data are mean ± SEM. **P* < .05 vs sham-sham, #*P* < .05 vs asphyxia-vehicle, &*P* < .05 vs 2 hour human amniotic epithelial cell (hAEC) group. ×20 magnification, scale bar = 50 μm

2 and 24 hour hAEC groups (*P* < .05). There was no significant difference between the hAEC groups.

4 | DISCUSSION

This study demonstrates for the first time that both early (2 hour) and late (24 hour) intracerebroventricular hAEC administration after severe asphyxia in preterm fetal sheep were associated with improved myelination and hippocampal neuronal survival. Interestingly,

administration of hAECs at 24 hours but not 2 hours after UCO was also associated with increased numbers of MBP-positive oligodendrocytes and improved neuronal survival in the striatum and thalamus compared to asphyxia-vehicle. Moreover, both groups showed consistent suppression, to sham control values, of the microglial reaction in white and gray matter and of astrocytosis in white matter. However, cell administration did not attenuate the overall loss of oligodendrocytes in the intragryal or periventricular white matter in either hAEC group and did not improve recovery of EEG power or spectral frequency. The key finding, that the overall histological improvement

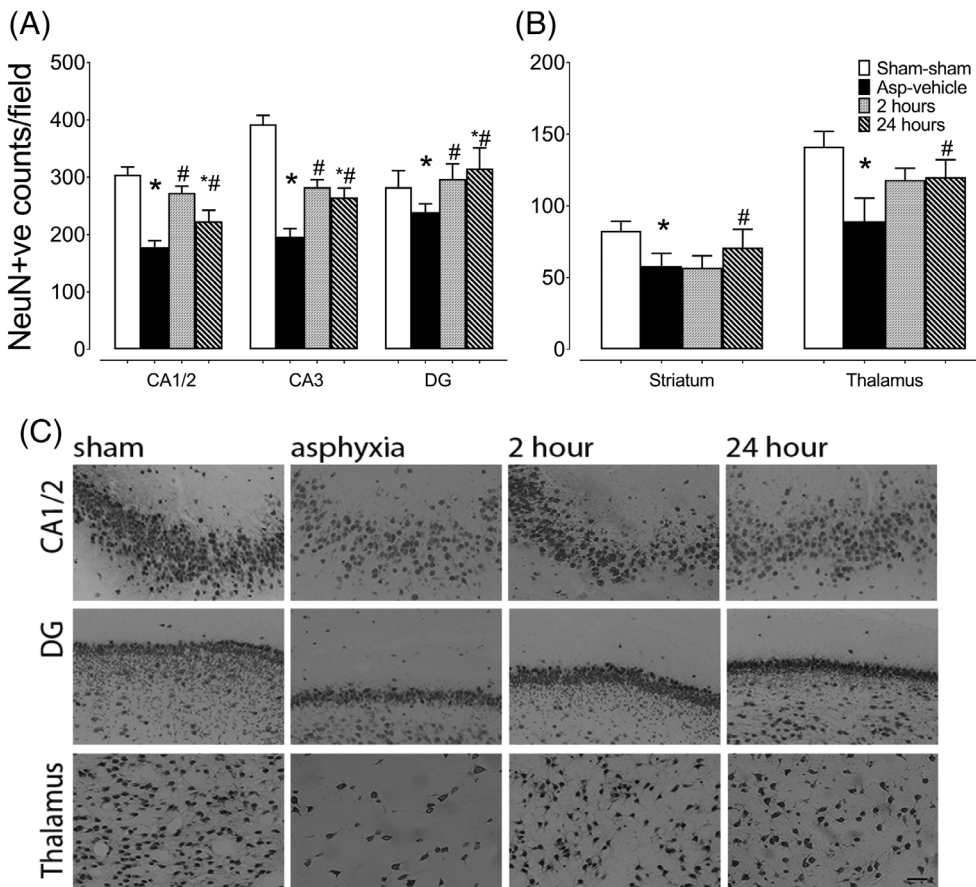


FIGURE 4 Neuronal survival after asphyxia. Changes in neuronal nuclear antigen (NeuN)-positive neuronal number assessed in the cornu ammonis 1/2 and 3 (CA1/2, CA3) and dentate gyrus (DG) of the hippocampus (A) and the striatum and thalamus (B) in the sham-sham (white), asphyxia-vehicle (black), 2 hour treatment group (gray), and 24 hour treatment group (striped). Photomicrographs of the CA1/2, DG and Thalamus (C). Data are mean \pm SEM. * $P < .05$ vs sham-sham, # $P < .05$ vs asphyxia-vehicle. $\times 20$ magnification, scale bar = 50 μ m

with hAEC treatment after acute hypoxia-ischemia was sustained or even, in some respects, greater after delayed administration at 24 hours postocclusion has important implications for treatment of the preterm newborn, where timing of injury is often unclear,⁷ and may be chronic or related to multiple insults.³⁵⁻³⁷

hAECs are pluripotent, can engraft³⁸ and have the potential to differentiate into neurons, astrocytes, or oligodendrocytes,³⁹ which may functionally express neurotransmitters.⁴⁰ However, evidence to date suggests that in most situations their capacity to improve outcomes after hypoxic-ischemic injury is mediated by release of factors which support the inhibition of inflammation, the release of trophic factors such as NGF and BDNF which support cell survival, proliferation and development, and the release of anti-apoptotic factors.^{11,41} Consistent with this, only undifferentiated Stem-121-positive hAECs were seen in the periventricular white matter in the present study.

Exposure to hypoxia-ischemia induces significant neural inflammation that helps to clear injured cells and creates a microenvironment amenable for neurorepair.³ Injury primes microglia and astrocytes as resident immune cells within the brain, and their activation after hypoxia-ischemia facilitates release of increased levels of pro-inflammatory cytokines and chemokines, which are in turn associated with neural injury.^{3,42} Furthermore, several studies have shown that blood-brain barrier integrity is compromised in the hours to days after hypoxia-ischemia in the fetus and newborn allowing blood-borne immune cells to infiltrate the brain and augment the

neuroinflammatory response.⁸ hAECs have been shown to suppress multiple aspects of the innate and adaptive immune systems, including activation of neutrophils, macrophages, and to reduce T- and B-cell proliferation.^{18,41,43,44}

In the present study, increased numbers of microglia in the white and gray matter and astrocytosis in the white matter persisted 7 days after asphyxia. hAEC administration at both 2 and 24 hours was associated with a marked reduction in both glial cell types to sham-sham levels. Similarly, Li and colleagues found that umbilical cord blood cells administered at 12 hours, but not 5 days, after asphyxia in preterm fetal sheep was associated with improved myelination as measured by CNPase, in association with reduced numbers of microglia, although the effect on overall oligodendrocyte survival and astrogliosis was not reported.⁴⁵ Further supporting a general anti-inflammatory effect of hAECs, Yawno and colleagues found that, in preterm fetal sheep, hAEC treatment after lipopolysaccharide administration markedly reduced intracerebral microglial induction.¹⁹

It is now well known that cell death evolves over time after acute hypoxia-ischemia.⁴⁶ After the initial restoration of intracerebral oxygenation after the end of asphyxia, there is transient recovery of oxidative metabolism in a latent phase, for up to around 6 hours, followed by a progressive secondary failure of mitochondrial function that is associated with delayed seizures, and ultimately cell death.²⁵ In practice, the latent phase is the primary window of opportunity for many neuroprotective interventions, particularly

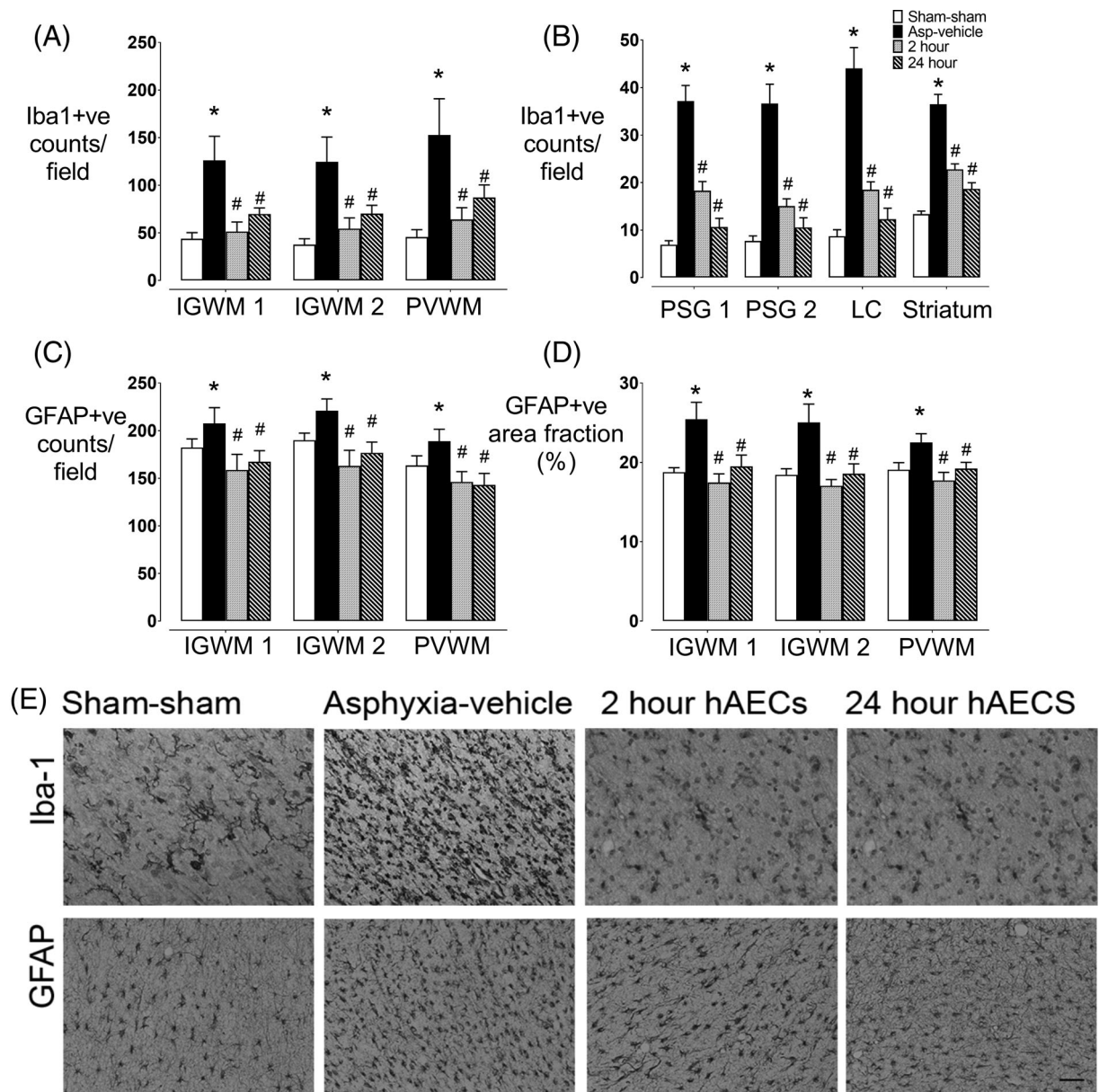


FIGURE 5 Numbers of microglia and astrocytes after asphyxia. Changes in ionized calcium binding adaptor molecule 1 (Iba-1)-positive microglia in the intragrayal white matter of the first and second parasagittal gyri (IGWM 1 and IGWM 2, respectively) and the periventricular white matter (PVWM) (A). (Iba-1)-positive microglia in the cortex of the first and second parasagittal gyri (PSG 1 and PSG 2 respectively), lateral cortex (LC) and striatum (B). Anti-gial fibrillary acidic protein (GFAP)-positive astrocyte number (C) and area fraction of labelling (D) in IGWM1, IGWM2, and PVWM. Sham-sham group (white), asphyxia-vehicle group (black), 2 hour treatment group (gray), and 24 hour treatment group (striped). Example photomicrographs of Iba-1-positive microglia and GFAP-positive astrocytes in the PVWM. Data are mean \pm SEM. * $P < .05$ vs sham-control, # $P < .05$ vs asphyxia-vehicle. $\times 20$ magnification, scale bar = 50 μ m

therapeutic hypothermia. Although hypothermia is not used clinically for treatment of preterm infants, in preclinical studies using the same protocol as the present study, prolonged cerebral hypothermia started at 30 or 90 minutes after UCO was highly protective,^{47,48} whereas white and gray matter protection was largely lost when initiation of hypothermia was delayed for 5 hours.⁴⁸ The finding that delaying hAEC administration until 24 hours after UCO, at a time when the secondary deterioration of mitochondrial function is well established in this model,²⁵ improved myelination and overall

neuronal survival denotes that cell therapy must be targeting different mechanisms compared to hypothermia.

It is notable that despite the profound reduction in astrocyte and microglial number in the white matter after hAEC treatment in the present study, there was no improvement in numbers of either total or immature/mature oligodendrocytes in the periventricular or intragrayal white matter. Intriguingly, we have recently shown that systemic TNF inhibition with Etanercept during LPS exposure in the preterm fetal sheep, also markedly attenuated white matter gliosis but did not

improve oligodendrocyte survival.³³ It is plausible that a reduction in the inflammatory state of the brain could be associated with improved function in surviving mature oligodendrocytes, despite no improvement in overall cell survival or restorative proliferation. If this hypothesis was correct, the significant increase in MBP area fraction and therefore myelination, despite no improvement in total or immature/mature oligodendrocyte number, may be due to improved function of mature oligodendrocytes after treatment with hAECs or direct protection of the myelin itself. Supporting this speculation, inhibition of microglial activation with minocycline administration after global cerebral ischemia in mice reduced loss of myelin basic protein.⁴⁹

There were small apparent differential effects of treatment timing on neuronal survival in the present study. Both the 2 and 24 hour hAEC treatment groups showed significantly improved neuronal survival in the hippocampus, however, hAEC administration at 2 but not 24 hours was associated with improved neuronal survival in the CA1/2 region to sham-sham levels. By contrast, the 24 hour, but not 2 hour, hAEC group showed improved neuronal survival in the striatum and thalamus compared to asphyxia-vehicle. These apparent regional differences in protection with timing of administration may reflect the temporal progression of injury as reported by others and targeting of stem cells to those regions.⁵⁰ For example, mesenchymal stem cells (MSCs) given at days 1, 4, and 7 days to adult rats after middle cerebral artery occlusion, showed that cells migrated to different regions depending on the day of administration.⁵¹ Alternatively, it might relate to the timing of migration of stem cells to injury sites. A study of mesenchymal stem cells given intranasally to postnatal day 9 (P9) mice 10 days after hypoxia-ischemia found that stem cells reached injury sites within 2 hours, and that their number decreased after around 12 hours.⁵²

Despite the improved neuronal survival, hAEC infusions did not significantly improve recovery of EEG power or spectral edge frequency in either treatment group compared to asphyxia-vehicle. Intriguingly, early hAEC treatment was associated with a trend toward greater EEG suppression between 30 and 42 hours postocclusion and a significant reduction of interictal EEG activity at 35 hours postocclusion. However, this was not correlated with neuronal survival or CaBF. Finally, we observed that administration of hAECs at 2 hours was associated with a significant reduction in interictal EEG power. This finding is consistent with recent studies in fetal sheep of administration of mesenchymal stromal cell-derived extracellular vesicles⁵³ and MSCs after asphyxia.⁵⁴ The mechanisms of this EEG suppression by hAECs between seizures are unknown, but likely multifactorial and may include reduced inflammation, and an altered balance between inhibitory and excitatory neurotransmitters and neuromodulators, and suppression of metabolism.^{55,56}

Alternatively, the transient effects of hAECs administered at 2 hours on EEG power may be mediated through changes in neurotransmitters such as dopamine. Data suggest that hAECs express dopamine (D2) receptors and recent data show that these receptors can downregulate glutamate neurotransmission and excitotoxicity, reduce seizures, and protect the hippocampus after kainic acid injections.⁵⁷ hAECs may also increase release of inhibitory

neurotransmitters such as catecholamines and acetylcholine.³⁹ In a mouse model of maple syrup urine disease, hAECs normalized metabolism and reduced deregulation of serotonin, dopamine, and gamma-aminobutyric acid (GABA) activity.⁵⁸ Furthermore, hAECs also support release of trophic factors,^{10,11} which may modulate seizure activity.

Consistent with the current study, we have previously shown that intranasal administration of hAECs given at 1, 3 and 10 days following 25 minutes of complete umbilical cord occlusion in preterm fetal sheep was associated with reduced microglial and astrocyte number and improved neuronal survival in the hippocampus, striatum, and thalamus after 21 days recovery.²³ However, in the previous study, hAEC administration was associated with restoration of immature/mature oligodendrocytes, whereas in the current study no effect on oligodendrocyte survival was seen. The differential effect on oligodendrocyte survival may be due to number and timing of treatments with hAECs. It is feasible that repeated doses of hAECs may be more beneficial than a single dose, but this has not been directly tested in the preterm fetal sheep. Alternatively, it is possible that the route of administration of hAECs could affect outcomes, for example, if peripheral immune modulation was needed for part of the effect of stem cells.⁴⁵

Limitations of this study include a sex imbalance in the 24 hour hAEC group, which had predominantly more males despite randomization of fetuses to treatment groups. It has previously been shown that some male fetuses may have different hemodynamic adaptations to asphyxia than females.⁵⁹ Furthermore, the use of an intracerebroventricular route of administration for hAEC delivery is less clinically translatable than intravenous or intranasal routes. In the present study, we chose intracerebroventricular administration to achieve a highly localized CNS response without altering the systemic inflammatory response and to avoid any confounding effects of altered brain blood flow or cardiac output with time after asphyxia.⁶⁰ Finally, brain injury can continue to evolve for many weeks and months after injury, and so it will important to assess the long-term outcomes in future studies.²³

5 | CONCLUSION

This study shows that in preterm fetal sheep, hAECs given at either early (2 hours) or delayed (24 hours) times after asphyxia, similarly attenuate neuronal loss and microglial and astrocyte upregulation and partially improve myelination. However, hAEC administration was not associated with improved oligodendrocyte survival or recovery in EEG power or spectral edge frequency. The observation that administration at 24 hours showed similar efficacy to administration at 2 hours suggests that hAECs have a much wider therapeutic window than therapies such as therapeutic hypothermia and so have potential for treating preterm infants who have been exposed to hypoxia-ischemia before birth. Further studies of the long-term recovery after cell therapy protocols in translational models are now indicated.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the technical assistance of Mrs. Rani Wilson and Mr. Vaho Maisashvili. This work was supported by the Health Research Council of New Zealand (grant numbers 12/613, 17/601). Lotte G. van den Heuij was an Auckland Medical Research Foundation Doctoral Scholar (grant number 1211003). Joanne O. Davidson is supported by a Sir Charles Hercus research Fellowship from the Health Research Council of New Zealand (16/003). Suzanne L. Miller and Graham Jenkin acknowledge the support of the Victorian Government's Operational Infrastructure Support Program.

CONFLICT OF INTEREST

Rebecca Lim declares employment with Monash University and a consultant/advisory role with Meluha Capital. All of the other authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

J.D., L.v.d.H.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing, final approval of the manuscript; M.F.: collection and/or assembly of data, final approval of the manuscript; G.W.: data analysis and interpretation, final approval of the manuscript; S.M., R.L., E.W., G.J.: provision of study materials or patients, final approval of the manuscript; A.G., L.B.: conception and design, data analysis and interpretation, final approval of the manuscript.

DATA AVAILABILITY STATEMENT

All data are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Davidson JO, van den Heuvel LG, Fraser M, et al. Window of opportunity for human amnion epithelial stem cells to attenuate astrogliosis after umbilical cord occlusion in preterm fetal sheep. *STEM CELLS Transl Med*. 2021;10:427-440. <https://doi.org/10.1002/sctm.20-0314>