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Neurologic outcomes of the premature lamb in an extrauterine environment for neonatal development☆,☆☆,★

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ABSTRACT

Background/Purpose: Neurologic injury remains the most important morbidity of prematurity. Those born at the earliest gestational ages can face a lifetime of major disability. Perinatal insults result in developmental delay, cerebral palsy, and other profound permanent neurologic impairments. The EXTracorporeal Environment for Neonatal Development (EXTEND) aims to transition premature neonates through this sensitive period, but it's impact on neurologic development requires analysis.

Methods: Fetal sheep were maintained in a fluid-filled environment for up to 28 days. Physiologic parameters were measured continuously; tissues were subsequently fixed and preserved for myelin quantification, glial cell staining, and structural assessment via magnetic resonance. Surviving animals were functionally assessed.

Results: No evidence of fetal brain ischemia or white matter tract injury associated with the EXTEND system was detected, and the degree of myelination was regionally appropriate and consistent with age matched controls. No evidence of neurologic injury or immaturity was visible on magnetic resonance; animals that transitioned from the system had no persistent neurologic deficits.

Conclusions: No evidence of major neurologic morbidity was found in animals supported on the EXTEND system, though more work needs to be done in order to verify its safety during critical periods of neurologic development.

Preterm birth and its complications remain a leading cause of death and significant morbidity in the developed world [1]. As recently as 2011, infant deaths related to prematurity were complicated by central nervous system (CNS) injury in as many as 18% of cases, with direct causation in 9%. [2] Neurodevelopmental complications are often permanent and impact quality of life much more significantly than other co-morbidities. [3] Despite decreases in mortality over the past two decades, no improvement in the rates of severe neurologic disability and neurodevelopmental impairment have followed; the burden of disease remains extremely high. [4–7] Rates of intracerebral hemorrhage and periventricular leukomalacia have decreased in infants 26 weeks or older, but no significant changes have been observed in neonates born at the youngest viable gestational ages. [8] The societal impact of preterm birth is also substantial. In 2007, The Institute of Medicine estimated that the cost of prematurity in the United States was $26.2 billion annually, including special education needs and loss of productivity, which accounted for up to $7 billion; [9] the individual cost over one-year for medical therapy alone was estimated at approximately $90,000 USD. [10]

The etiology of neurologic impairment in prematurity is complex and multifactorial, as brain injury results from numerous perinatal insults and disruption of complex maturational processes. [11] The entire constellation of pathologic findings is known as encephalopathy of prematurity, (EoP) a broad entity encompassing most central nervous system and developmental abnormalities associated with preterm birth. Development of preventative measures is especially difficult because of its multifactorial nature and the large number of processes that can be disrupted. For example, myelination is critical for efficient conduction throughout the central and peripheral nervous systems; the process spans from the second trimester of gestation through the second

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and third decades of life, with the most rapid changes occurring during the developmental window interrupted by premature birth. [12] An ideal treatment strategy for prematurity therefore would not only need to avoid damage to important CNS structures, but also promote normal maturational progression. Many currently available preventive strategies focus primarily on avoiding preterm birth altogether [13] or preventing serious insults, like seizures or inadequate ventilation. [14] As the rate of preterm birth in the developed world continues to rise, [15] a new paradigm in therapy is needed to reduce these burdens.

We recently reported the creation of an EXTrauterine Environment for Neonatal Development, (EXTEND) a system consisting of a pumpless oxygenator circuit, an umbilical vascular interface, and a fluid-filled womb-like environment. The EXTEND system allows for long-term physiologic support of premature lambs by preserving fetal circulatory pathways, thus obviating the need for harmful ventilation and exposure to other perinatal insults [16]. Fetuses placed on EXTEND (at the lung maturational equivalent of a 22–24-week gestation human) had comparable brain weights and histologic appearance to age matched controls. While these findings were encouraging, our primary focus up to that point was to achieve physiologic support and lung maturation over a 3–4-week period. Here we present our complete neurologic outcomes and analysis of fetal lambs maintained on and transitioned from EXTEND. We hypothesized that myelination in these animals would be regionally appropriate for gestational age, and that no significant evidence of injury or hypoxia would be evident on immunohistochemistry, imaging, or functional analysis. Though brain development on EXTEND is outside of the window of increased brain sensitivity, our primary goal is to demonstrate neurologic safety in the animals and experiments completed thus far.

1. Methods

1.1. Study design and animal selection for neurologic assessment

Eighteen experimental animals maintained on EXTEND and either analyzed by neurologic tissue histology or functional assessment/ MRI. (Supplemental Table S1, Supplemental Fig. S1) Six fetal lambs (GA, gestational age 105–117 days) supported by the EXTEND system for up to 4 weeks (as previously reported [16]) were selected for histologic staining. Briefly, animals maintained within the system are delivered via hysterotomy and placed within a plastic, fluid-filled enclosure after cannulation of two umbilical arteries and one umbilical vein at the distal-most aspect of the ovine umbilical cord; the oxygenator circuit is concomitantly opened and maintains full gas-exchange and nutritional support for the animal for duration of the study. Of the group of eight previously published animals, two were excluded due to the presence of Tetralogy of Fallot in one animal and ventilation difficulties leading to hypoxia post-therapy in another. Four histologic control animals were harvested via hysterotomy at GA 141; both experimental and control animals were briefly ventilated/euthanized in a similar manner, with prompt brain tissue procurement, fixation and subsequent analysis for histologic comparison. As previously reported, these animals had comparable fetal oxygen delivery, myelination, and an absence of histologic pathology in comparison to controls; [16] we therefore sought to further define the relationship between fetal oxygen delivery and brain vasculature, assess the degree of myelination based on the time spent on the circuit and final gestational age, and to further assess for any evidence of neuropathology within tissue specimens via immunohistochemistry.

Functionally assessed animals (including one lamb supported using an earlier prototype of the system – Carotid Artery Jugular Vein Cannulation, CA/JV 4 in the previous manuscript) were weaned from post-therapy ventilation or euthanized immediately for preservation and imaging. These included eight additional animals not previously reported. (Exp.7–14) Two of these animals underwent live-MRI after reaching at least 6 months of age, (CA/JV 1, Exp. 10) while the others were perfusion fixed and imaged immediately after euthanasia. Seven additional control animals were harvested via hysterotomy at GA 128 and 136; the brains were perfusion fixed in situ for imaging comparison at the immediate post-EXTEND therapy ages ranging from GA 129–135. No histologic analyses were carried out on these tissues due to the need for a different in situ fixation protocol for MRI. Pre-defined end-point euthanasia criteria were used for post-therapy animals transitioning from the EXTEND system, with a total experimental time of at least 3 weeks plus 1–2 days of post-therapy ventilation. No other outliers were excluded from any analysis reported.

Additional animals were used to characterize the progression of myelination on the EXTEND system; pre-EXTEND therapy control lambs ($n = 3$) were harvested at GA 100–115 and sectioned/preserved as detailed below. Experimental animals cannulated without completing full therapy runs (less than 3–4 weeks) were used to assess and illustrate the degree of myelination during therapy, as depicted in Fig. 3. (GA 114 and 125, $n = 2$) Further histologic analysis was not performed on these animals due to their relative immaturity and other confounding factors. In order to characterize neurologic function while on circuit, an additional six animals (GA 95–119) were also cannulated and observed twice daily for up to 8 days while on circuit.

All studies were conducted with the approval of the institution's animal care and use committee at the Children's Hospital of Philadelphia, and in accordance with the NIH Guide for the Care and Use of Laboratory Animals (8th Edition) and the Animal Welfare Act and other federal statutes regulating animal experimentation.

1.2. Preservation and sectioning for histologic analysis

Brains from experimental animals 1–6 and controls 1–4 were submerged in 10% formalin (EMD Millipore Corp, Billerica, MA, USA) for 7–10 days and then sectioned and placed in paraffin prior to mounting and staining. Tissues were sectioned into anterior and posterior cortical regions for each hemisphere. Hematoxylin and eosin and Kluver and Barrera Luxol fast blue staining was performed as detailed in Laboratory Methods in Histotechnology (Armed Forces Institute of Pathology, Washington, DC) [17] Slides were then digitally scanned at 20X magnification and evaluated using Aperio Imagescope Version 12.3 (Leica Biosystems Pathology Imaging, Buffalo Grove, IL, USA) and standard light microscopy.

1.3. Digital staining and morphometric quantification

For morphometric cerebral arteriolar assessment, the diameter of at least 30 cortical arterioles from each experimental animal (1–6) and their controls were measured digitally using the ruler function in Aperio Imagescope. (Leica) The cross-sectional area of each vessel was then calculated using the formula:

$$A = \pi(d/2)^2$$

where A = cross-sectional luminal area, d = diameter of the vessel.

Myelination density was quantified digitally in experimental animals 1–6 and controls 1–4 using a method similar to one described by Lairson et al. [18] Myelin-positive pixel ratios were calculated using Positive Pixel Count Version 9 (Aperio Technologies, Leica Biosystems) A single algorithm was used to identify pixels (within outlines of the various white matter tracts of the brain) consistent with positive staining in both groups. Percent positivity within each outlined region was used as a surrogate of the degree of myelination and was defined as the percentage of pixels consistent with positive staining for myelin out of the total number of pixels within the outlined field.

1.4. Immunohistochemical staining and quantification

Experimental tissues 1–6 and control tissues 1–4 were assessed using immunohistochemistry, including Polyclonal Rabbit anti-Glial Fibrillary Acidic Protein (Z0334; Dako, Agilent Technologies, Santa Clara, CA, USA) at 1:200 dilution and Anti-Iba-1 antibody (ab5076; Abcam, Cambridge, MA, USA) at 1:1000 dilution. All antibodies were diluted using Dako antibody diluent. The slides were covered with antibody and incubated overnight at 4 °C. After rinsing in TBS, slides were incubated with secondary antibody for 10 min at room temperature (Super Picture HRP Polymer Conjugate, Broad Spectrum; Life Technologies) followed by rinsing in TBS. Stains were then developed using DAB (SK4105, Vector Labs). Brown spots under light microscopy indicated the presence of each specific protein.

Quantitative immunohistochemistry (IHC) analysis was performed using separate uniform algorithms (Positive Pixel Count and Cell Count, Leica Biosystems) adjusted to identify pixels and nucleated cells consistent with positive IHC staining for each individual region outlined. Positivity in the pixel-based algorithm was defined as the number of positive pixels within the defined area divided by the total number of pixels. Positive cells were defined as nucleated cells with positive staining. Average intensity was calculated as the intensity of positive pixels divided by the total number of positive pixels within a defined area.

1.5. Magnetic resonance imaging

Experimental animals 7–9, 11–14 (as well as an additional two animals euthanized after 9 days of therapy) and MRI controls 1–7 underwent prompt perfusion fixation after euthanasia via the common carotid artery with a 10% formalin infusion; the decapitated heads were then maintained in 10% formalin for 7–10 days, after which MRI was performed. All MRI examinations were performed on a 3-T Siemens MAGNETOM Trio MRI system (Siemens, Malvern, PA). Two MRIs were performed on live animals (CA/JV 1 and Exp. 10) sedated with ketamine (4–5 mg/kg) and midazolam, (0.3–0.5 mg/kg) followed by Propofol (2–8 mg/kg) for induction, and isoflurane (1–5%) for maintenance. Sequences included T1-and axial T2-weighted imaging, as well as T1 volumetric quantification in experimental animals 11–14 and their respective controls. MRI examinations were blindly reviewed by a neuroradiologist and a pediatric radiologist and were evaluated for structural abnormalities, evidence of white matter injury, and maturation of the myelination pattern. Diffusion tensor imaging could not be performed due to timing and resolution limitations.

1.6. Statistical analysis

Myelination densities, IHC staining positivity, and comparison of other continuous variables were performed using Student's unpaired *t*-test (GraphPad Prism Version 6, GraphPad Software, San Diego, CA, USA). Significance was accepted at $P < 0.05$. All data are presented as mean ± SEM. The relationship between fetal oxygen delivery and cerebral arteriolar cross-sectional area was assessed by Pearson's correlation coefficient, (GraphPad Prism) whereas the degree of myelination by gestational age and middle cerebral artery resistance indices by gestational age were assessed via linear regression analysis. (GraphPad Prism).

2. Results

2.1. Cerebral perfusion is maintained on EXTEND

All animals included in the study are listed in Supplemental Table S1, and the subset of lambs that were assessed histologically are detailed in Table 1. As previously reported, no evidence of neurologic insult (ischemic changes, demyelination) or hemorrhagic bleeding was found on H&E staining. Because inadequate brain perfusion has been shown in fetal lambs to cause cerebral arteriolar dilation, [19] we measured the cross-sectional area of cerebral arterioles in the frontal and parietal sections of the cortex. (Fig. 1A) No significant differences in size between experimental animals and control animals were identified in any region examined. (Fig. 1B) Additionally, the mean luminal area (of all four quadrants combined) for each animal was negatively correlated with the mean fetal oxygen delivery calculated across the duration of the cannulation, ($r = 0.93$, $p = 0.02$) indicating cerebrovascular responsiveness to oxygen availability; (Fig. 1C) Experimental lamb 1 was excluded from this correlation due to differing oxygen delivery measurement and management from an earlier prototype of the EXTEND system. The middle cerebral artery resistance index (a surrogate for resistance to flow distal to the point of measurement) at 130 days gestation was equivalent to measurements published by Arbeille et al. [20] (Fig. 1D) and showed the expected decrease towards the end of gestation. ($r = 0.44$, $p < 0.0001$) (Fig. 1E).

2.2. Brain parenchyma does not show evidence of neurologic injury

Fig. 2 depicts representative glial staining of each of the six animals evaluated by a veterinary neuropathologist. Of all fetal sheep maintained on circuit, none displayed Iba-1 glial activation and staining suggesting discrete injuries or chronic hypoxic insult in relation to controls.

Table 1
Experimental animals by histologic brain staining and evaluation.

	Final gestational age (days)	Time on circuit (days)	Hematoxylin/ eosin	Luxol fast blue	Iba-1 IHC	GFAP IHC
Exp. 1	134	26	nl	nl	1 of 4 slides mod. Incr. WM glia	nl
Exp. 2	138	28	nl	nl	nl	nl
Exp. 3	136	20	nl	nl	nl	1 of 4 slides mod. Incr. # of astrocytes internal capsule/ thalamus
Exp. 4	144	24	nl	nl	nl	1 of 4 slides mild incr. # of astrocytes internal capsule/ thalamus
Exp. 5	132	20	nl	nl	nl	nl
Exp. 6	140	28	nl	nl	nl	nl
EXTEND MEAN	137 ± 4	24 ± 4				
Control 1	141	N/A	nl	nl	nl	nl
Control 2	141	N/A	nl	nl	nl	nl
Control 3	141	N/A	nl	nl	nl	nl
Control 4	141	N/A	nl	nl	nl	nl
CONTROL MEAN	141 ± 0	N/A				

nl = normal findings without evidence of ischemic, hemorrhagic, or white matter tract injury.

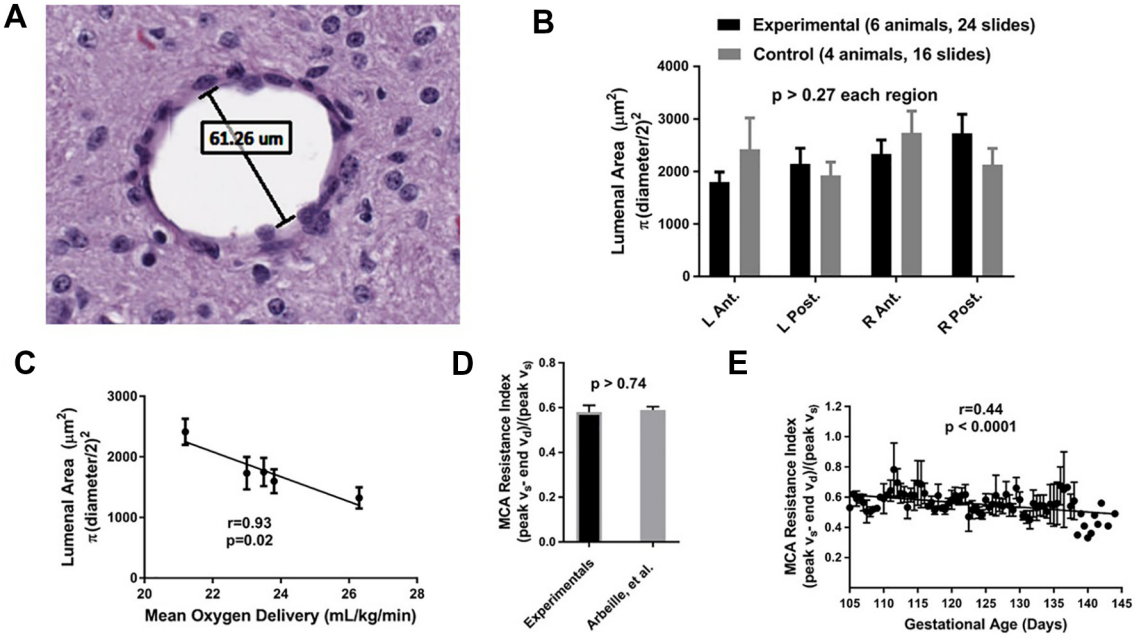

Fig. 1. Cerebrovascular morphology and function on EXTEND. **(A)** Representative H&E stained cortical arteriole with the measured diameter displayed in the center. **(B)** Mean cross-sectional area of arteriolar lumens measured in each of the 4 quadrants of the brain. **(C)** Relationship of arteriolar area to mean oxygen delivery for each animal maintained on circuit; Exp. 1 was excluded due to differences in measurement and management. (Pearson's correlation coefficient, $r = 0.93$, $p = 0.02$) **(D)** Middle cerebral artery resistance index (MCA RI) by ultrasonography at 130 days gestation compared to Arbeille et al. [20] **(E)** MCA RI measurements on EXTEND by gestational age. (linear regression, $r = 0.44$, $p < 0.0001$).

(Fig. 2A, B, G, H) Additionally, digital staining quantification in the cerebrum, basal ganglia, and cerebellum showed no significant difference between experimental and control tissues. (Fig. 2K) To further assess for glial activation, glial fibrillary acidic protein (GFAP) immunohistochemistry was performed on each of the sections as well; (Fig. 2C, D, I, J) GFAP can be upregulated and strongly expressed by astrocytes exposed to insults within the CNS. Only one out of four slides in experimental lambs 3 and 4 showed an increased number of astrocytes. Digital quantification of these stains revealed no significant differences from control tissues in each region of the brain evaluated. (Fig. 2L) There was also no statistical difference in the total percentage of cerebral nuclei staining positive for Iba-1 (Fig. 2M) or GFAP (data not shown.) All stains were also compared in terms of intensity in order to rule out technical error or differences in staining quality. (Supplemental Fig. S2).

2.3. Fetal myelination and maturation of white matter tracts

After finding no evidence of demyelinating lesions on Luxol fast blue stains, [16] we measured the amount of myelin via digital densitometry within the corona radiata of pre-cannulation, mid-cannulation, and post-decannulation animals. (Fig. 3A) Myelin within this region was increasingly intense in representative mid and post-decannulation animals, (represented by dark orange staining to the right) while staining in pre-cannulation control animals was less intense. (GA100 and 115) Additionally, the final gestational age of the animals that completed a full course on circuit was directly related to the amount of myelin quantified in the same region. ($r = 0.87$, $p = 0.02$) (Fig. 3B) In order to assess myelination and maturation throughout the brain, we expanded our previous assessment [16] to include myelinated tracts from the cerebellum up to the cortical gyri. (Fig. 3C, D) Staining was not statistically different between experimental animals and controls in any region analyzed, though myelination the cerebellar arbor vitae trended higher and the cortical gyri trended lower in experimental animals.

2.4. Global brain assessment via magnetic resonance imaging

We next used MRI to evaluate evidence of parenchymal injury and the degree of myelination/maturation. Table 2 details the subset of circuit and control animals that were assessed. Experimental animals had no evidence of white matter injury, periventricular leukomalacia, or ischemic/hemorrhagic insults. In addition, the degree of myelination of animals imaged after placement on the system increased with increasing gestational age and continued after decannulation, as evidenced by the increasingly darkened areas within the central portions of the brains. (Fig. 4A) Animals which were harvested mid-treatment also demonstrated more mature myelination patterns than pre-cannulation controls suggesting continued myelination during treatment. Animals imaged from GA129–135 had myelination patterns similar to controls born at 128–136 days gestation, suggesting that the patterns are age appropriate. (Fig. 4B, D) T1 weighted volumetric scans also showed similar brain volume to biparietal diameter ratios within the same age-range. (Fig. 4C).

2.5. Neurologic function on and off circuit

In order to evaluate lamb activity on circuit, we cannulated an additional 6 animals in studies that were carried out for up to 9 days. (Fig. 5) The lambs remained active for the duration of the experiment. Lambs were actively moving in the fluid environment approximately 60% of the time when observed up to twice daily and fetal breathing frequency was comparable to previously published reports; [21, 22] (Fig. 5B). Experimental animals 1–6 also maintained circadian fluctuations in both heart rate and blood flow through the umbilical circulation across the duration of the four-week period, (Fig. 5C and D) and were found to produce their own melatonin free of maternal influence. (Supplemental Fig. S3) We next sought to assess the neurologic function of animals that were successfully extubated post-therapy, as detailed in Table 3. All animals were able to visually track, suckle, and vocalize shortly after transitioning out of the EXTEND device. Once extubated, animals were given physical therapy prior to each bottle feeding until they were able to walk independently. (Fig. 6) Lambs eventually grew strong enough to run and balance on all surfaces, which also enabled them to follow and seek out food and their caretakers. No evidence of persistent neurologic injury has been found in lambs that were transitioned from the ventilator, of which there are now four in total.

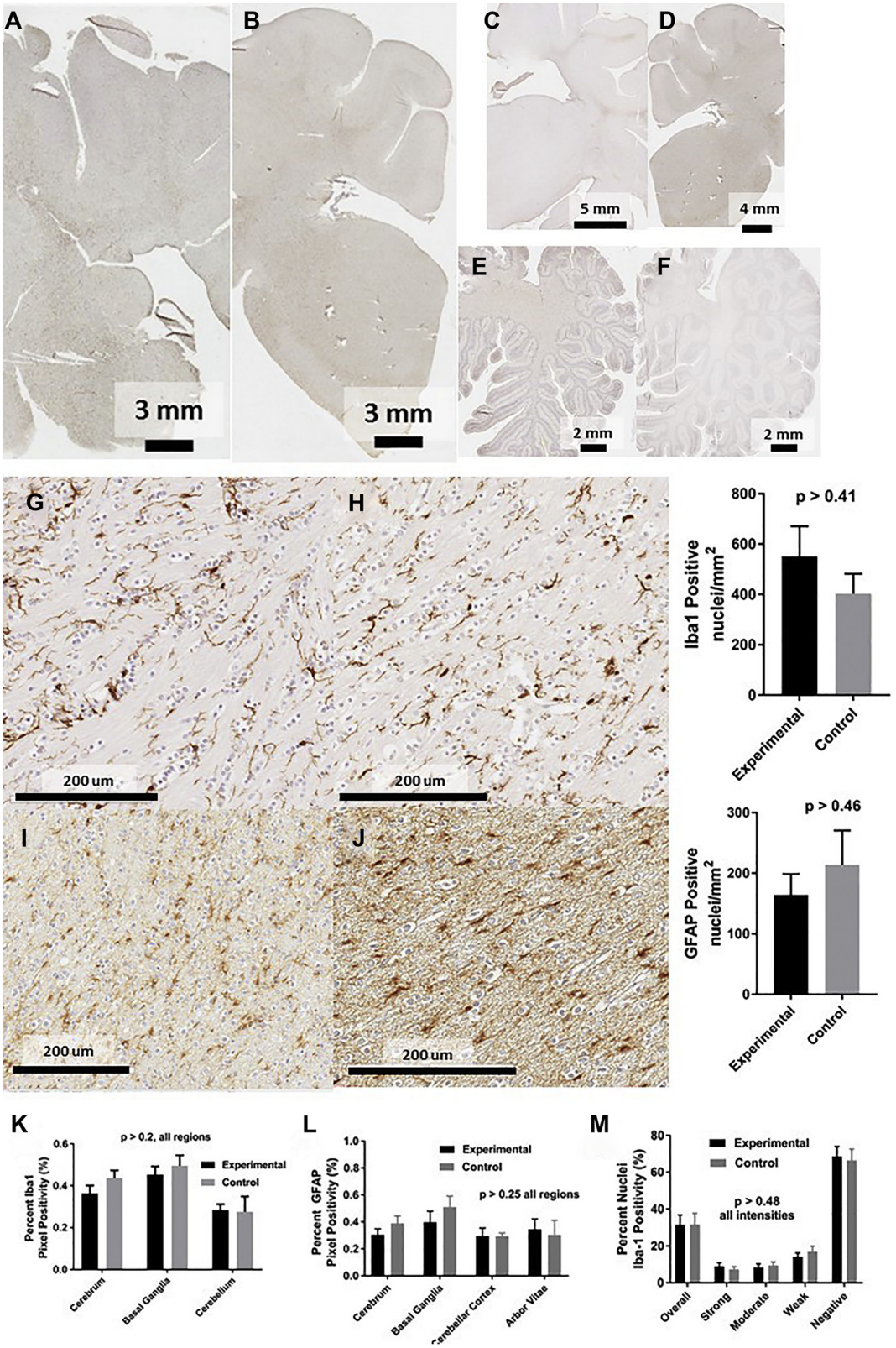

Fig. 2. Representative CNS glial cell staining and quantification. Iba1 immunohistochemistry staining in **(A)** experimental cerebral cortex, **(B)** control cortex. GFAP staining in **(C)** experimental cerebral and **(D)** control cortices. **(E)** Iba-1 staining in experimental and **(F)** control cerebellar cortices. **(G)** Iba-1 stains in experimental and **(H)** controls at high magnification within the corona radiata and the respective number of positively stained nuclei/mm^2. **(I)** GFAP immunostaining in experimental and **(J)** controls at high magnification within the corona radiata and the respective number of positively stained nuclei/mm^2. **(K)** Percent pixel positivity in Iba1 stains and **(L)** GFAP stains. **(M)** Percentage of nuclei staining positive for Iba-1.

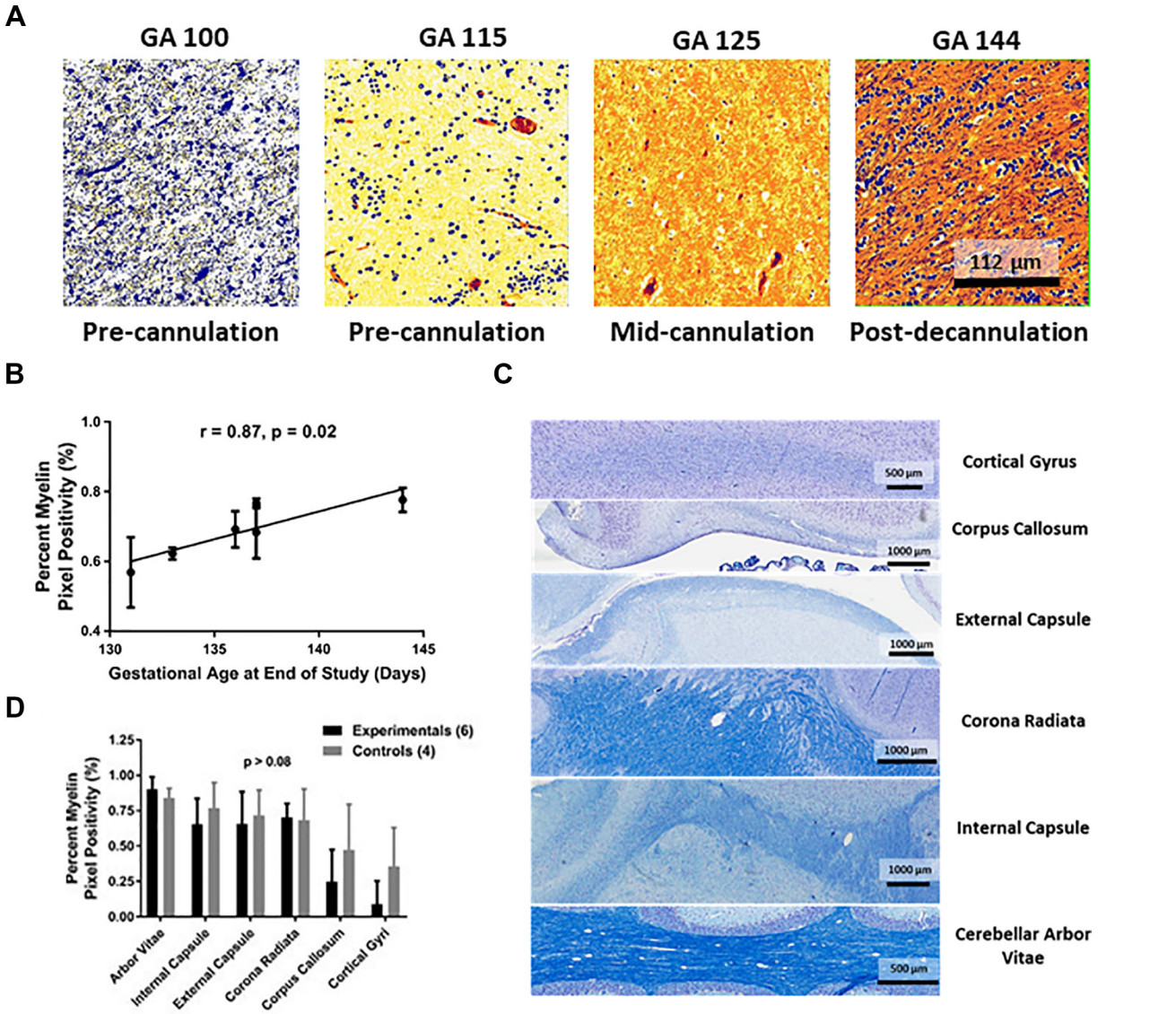

Fig. 3. Myelination and white matter maturation before, during, and after therapy. **(A)** Digitally identified myelin (yellow/orange) increasing within the corona radiata of representative sheep by gestational age. **(B)** Myelin densitometry by gestational age; (linear regression, r = 0.87, p = 0.02) **(C)** Representative Luxol fast blue stains for each white matter tract evaluated. **(D)** Digital myelin densitometry by brain region.

3. Discussion

Artificial oxygenation and support systems for preterm neonates have been under development for decades, and some groups have even reported neurologic findings from their studies. As recently as 2013, Gray et al. [23] found no evidence of intraventricular hemorrhage after 70 h of pumped veno-venous support in premature lambs, and up to 1 week of support in 2015, [24] though they did not report on white matter tissue integrity. The animals moved all limbs and were responsive to stimuli while on circuit. In contrast, Miura et al. [25] reported that two lamb fetuses supported on a parallel oxygenation circuit were found to have evidence of coagulation necrosis and cyst formation after approximately 60 h of circuit time, which they speculated was either due to systemic circulatory shunting through the circuit or hypovolemia associated with the cannulation procedure. Both groups utilized heparinized circuits but did not report any hemorrhagic complications.

Fluctuations in cerebral perfusion and oxygenation, especially in the setting of hemodynamic instability and autonomic regulatory immaturity may play a role in some brain lesions associated with prematurity. [26, 27] This study illustrates that the cerebral vasculature is sensitive to total fetal oxygen delivery, and that measures of vascular resistance are comparable to other reports in the literature. In addition, glial stains were not consistent with chronic oxygen deprivation or acute insult. Taken together with our previously reported finding of middle cerebral artery responsiveness to fetal oxygen delivery, [16] it appears that these fetuses regulate cerebral blood flow to the brain based on oxygen availability, and do not display evidence of cerebrovascular insufficiency or ischemia.

In the absence of overt signs of cerebrovascular injury, we turned our attention to look for more subtle findings within CNS cell populations. Glial cells (including microglia and astrocytes) can become activated and mobilized in the presence of physiologic stress or insult; they are also thought to play a role in the neurologic injury that is sustained in such instances. Iba-1, an actin-binding protein that is expressed and upregulated during microglial and macrophage activation, is a surrogate marker that can indicate the presence of neurologic injury. Similarly, GFAP is a protein expressed by astrocytes that can also be used for identification. Glial staining by light microscopy and digital quantification were congruent between groups, including Iba-1 microglial positivity and GFAP astrocyte positivity for both staining intensity and the percentage of nucleated cells present. It is difficult to interpret the moderately increased levels of staining on three of the slides

Table 2
Magnetic resonance imaging findings before, during, and after therapy.

Animal	GA at MRI	Findings (T1/T2)
CA/JV 1	6 mos. Post tx.	No MRI evidence of parenchymal injury
Exp. 7	137 + 12	No MRI evidence of parenchymal injury
Exp. 8	130 + 74	No MRI evidence of parenchymal injury
Exp. 9	145	No MRI evidence of parenchymal injury
Exp. 10	6 mos. Post tx.	No MRI evidence of parenchymal injury
Exp. 11	130	No MRI evidence of parenchymal injury
Exp. 12	135	No MRI evidence of parenchymal injury
Exp. 13	128	No MRI evidence of parenchymal injury
Exp. 14	129	No MRI evidence of parenchymal injury
Pre-therapy Ctrl 1	105	Shallow sulci/ immature myelination pattern
Pre-therapy Ctrl 2	108	Shallow sulci/ immature myelination pattern
Pre-therapy Ctrl 3	121	No MRI evidence of parenchymal injury
Mid-therapy 1	129	No MRI evidence of parenchymal injury
Mid-therapy 2	124	No MRI evidence of parenchymal injury
MRI Ctrl 1	128	No MRI evidence of parenchymal injury
MRI Ctrl 2	128	No MRI evidence of parenchymal injury
MRI Ctrl 3	136	No MRI evidence of parenchymal injury
MRI Ctrl 4	136	No MRI evidence of parenchymal injury
MRI Ctrl 5	136	No MRI evidence of parenchymal injury
MRI Ctrl 6	136	No MRI evidence of parenchymal injury
MRI Ctrl 7	136	No MRI evidence of parenchymal injury

analyzed, (out of a total of 24 experimental slides) especially given the lack of findings on matched sections that were stained with H&E, Luxol Fast Blue, and the corresponding opposite glial stain. Lambs that were transitioned from the EXTEND system earlier on in its development were intermittently extubated prior to euthanasia to allow for independent ventilation, which may have also introduced the possibility of hypoxic insult as oxygen saturation could only be intermittently monitored. An adequate comparison perhaps would be a group of extremely preterm lambs ventilated over a similar timeframe; however, lambs at 95–110 days gestation and earlier cannot fully oxygenate their blood when ventilated [28] and are not viable without extracorporeal support. These experiments were therefore not pursued. Regardless, digital cell counting and intensity quantification did not reveal any statistical difference between the two groups and staining patterns in the animals did not demonstrate chronic cerebral hypoxia or ischemic injuries. We are actively working to characterize glial migration and maturation (including oligodentrocyte maturation) within this system.

After analyzing myelinated tracts in a number of sheep across gestational ages, it is clear that the amount of myelin deposited within the corona radiata is higher in animals maintained on EXTEND than in pre-therapy and mid-therapy comparisons. Final gestational age was strongly, positively associated with myelin deposited in the same region, and myelin deposits were not statistically different in the other tracts assessed. Consistent with what is known about brain maturation, the most myelin was detected in the more "primitive" parts of the brain such as the cerebellum, whereas the least amount was found in the higher white matter tracts and cortical gyri. While myelin deposition within the higher tracts trended lower in the experimental animals, sheep myelination within the cerebral cortex does not reach full maturation until 140 days gestation, [29] and experimental lamb 4 (who reached 144 days gestation) was found to have similar levels of myelin staining within the same region when compared to controls. The trend in higher levels of the brain therefore is possibly due to non-pathologic immaturity rather than a failure to properly myelinate on EXTEND. The presence of myelin as assessed via MRI also increased with increasing gestational age and was comparable between age matched controls at GA129 and GA136, and no MRI evidence of white matter injury (demyelinated/scarred tracts) or other serious insults were found in any of the imaged animals. In addition, brain parenchymal volume measured on MRI was equivalent between groups, consistent with our previous finding of equivalent brain weight in relation to body weight. [16] In the presence of acute ischemic insults, injuries are typically evident on MRI within days, and chronic findings can certainly persist past that immediate period, [30] making it likely that any injury sustained over 4 weeks would be visible. Additionally, intracerebral hemorrhage and its sequelae were not visible on any of the scans performed, though

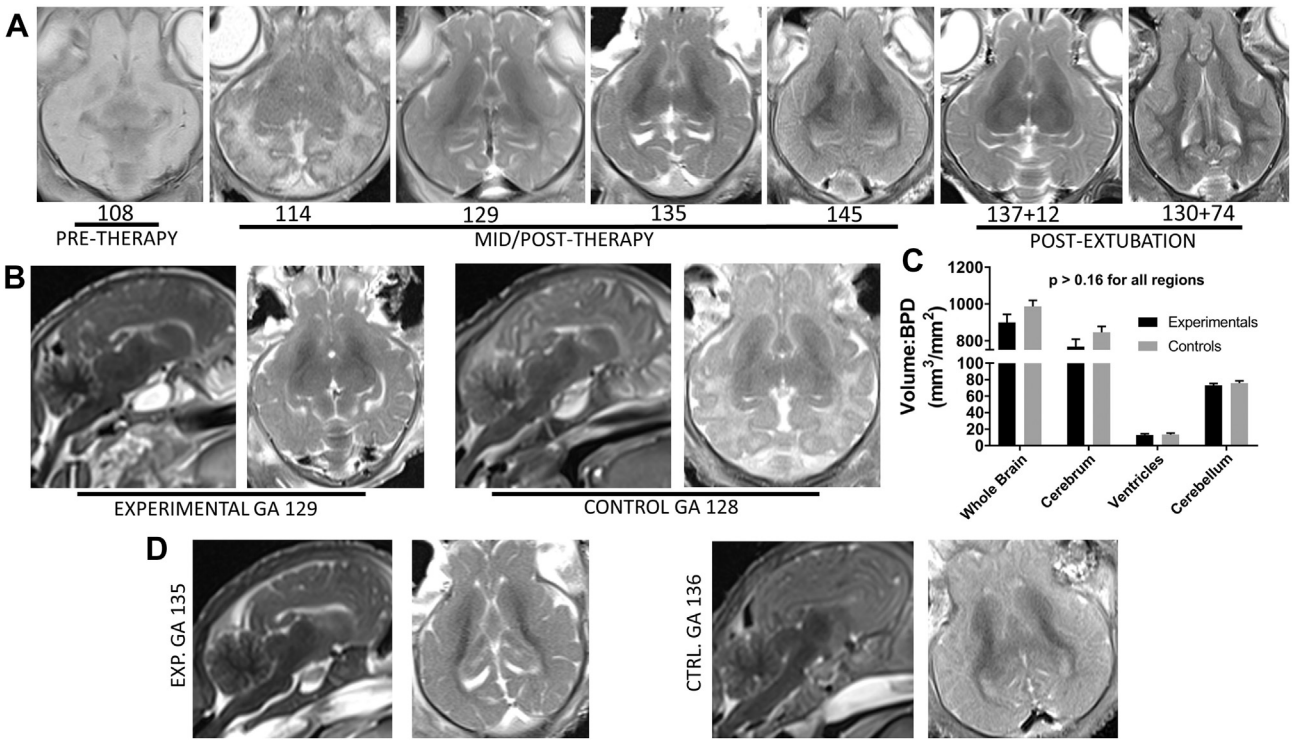

Fig. 4. Representative T2-weighted MRI images in control and cannulated animals. **(A)** Experimental animals demonstrating increasing levels of myelination as gestational age increases. **(B)** Sagittal and axial comparison of age-matched GA 128/129 experimental and control animals. **(C)** Brain parenchymal volume to biparietal diameter ratios in experimental vs. control animals, GA128–136. **(D)** Sagittal and axial comparison of age-matched GA 135/136 experimental and control animals.

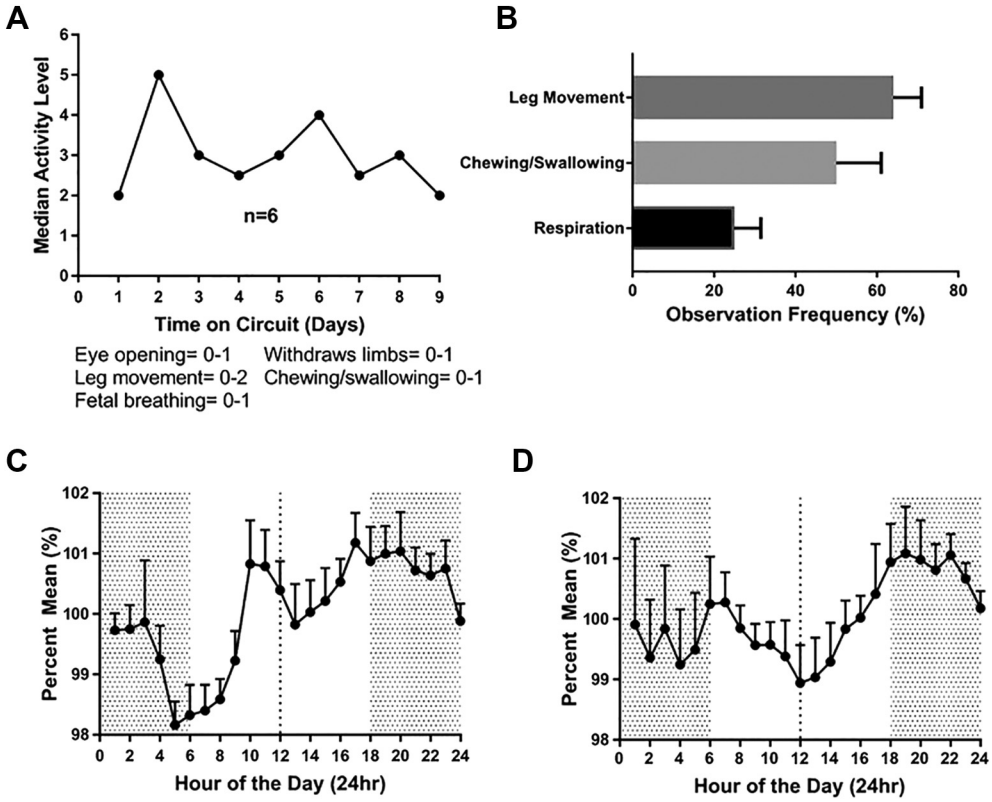

Fig. 5. Animal activity on circuit. **(A)** Median activity level as depicted by an ordinal scale in a separate cohort of 6 EXTEND animals maintained for up to 9 days. **(B)** Percentage of time leg movements, chewing/swallowing motions, and breathing activity were observed during daily observations. **(C)** Circadian heart rate plot represented as a percentage of the overall mean across a 24-h cycle averaged over the duration of therapy for experimental animals 1–6. **(D)** Circadian circuit blood flow plot.

germinal matrix hemorrhage would be unlikely given that these animals are already past the age of sensitivity at the time of cannulation. [31] In the absence of histologic or radiographic evidence of injury, our focus turned to functional outcomes.

Lambs on EXTEND display normal levels of fetal activity within the fluid filled environment and maintained circadian rhythms, free from any maternal influence or support. Once we were confident that lung development and function on the ventilator was age appropriate in experimental lambs 1–6, we next worked towards extubating our animals to allow for post-therapy neurologic assessment. While extubated lambs appeared deconditioned in comparison to term lambs, (GA 145–150) they gradually progressed from being unable to hold their heads up, (in some instances) to sprinting up and down the halls of the animal facility after approximately a week of daily physical therapy. (Supplemental Movie S1) No lambs displayed any evidence of permanent deficits, (paralysis, intellectual disability, blindness, etc.) and all were generally playful and responsive to vocal stimulation. Three of the four ventilated survivors reached complete independence and were able to feed themselves ad libitum once weaned from bottle feeds. While we do not fully understand the reasons why these animals are unable to walk immediately out of the Biobag, we believe it is likely multifactorial and warrants further investigation; a large part may also be due to their relative prematurity post-therapy.

There are many important limitations to our study. Sheep are more neurologically mature at birth out of necessity, as their survival depends on their ability to nurse. Humans are extremely dependent when born and continue significant myelination well after birth, making it likely that insults in the perinatal period may affect humans differently (and more profoundly) than sheep. Models of developing white matter injury indicate a window of susceptibility to injury of around 90–100 days gestation, [31] whereas the earliest our group has cannulated animals for a successful long-term experiment is a gestational age of 105 days. Even maturation of autonomic regulation occurs slightly earlier than most of the animals we cannulated, developing around 90 days gestation. [27] As others have pointed out, [24, 25] the ovine germinal matrix matures much earlier than in the human fetus [32] and is likely more resistant to hemorrhagic complications at the time of cannulation making the sheep model a poor model for intracranial hemorrhage. Much more work will need to be done to determine the risk of intracranial bleed on this system at younger gestational ages, as well as whether or not a heparinized circuit is required for long-term support. Finally, as neurodevelopmental delay and subtle intellectual disabilities appear to make up a significant portion of neurologic morbidity in patients born prematurely, further functional testing once more surviving lambs are transitioned into post-therapy life will be critical. We are actively working on and developing such

Table 3
Functional progression post therapy.

Lamb	GA at Cann. (days)	Time on circuit (days)	Tracking (DoL)	Suckle (DoL)	Vocal (DoL)	Head support (DoL)	Foreleg support (DoL)	Hind leg support (DoL)	Standing (DoL)	Walking (DoL)	Survival
CA/JV 1	122	12	0	0	0	1	2	2	3	5	>365 days
Exp. 7	108	29	0	0	1	2	2	7	7	8	Euth. at 12 days
Exp. 8	110	20	0	3	1	4	6	6	7	8	Euth. at 74 days
Exp. 10	113	22	0	0	0	0	1	1	6	9	Euth. at 6 mos.

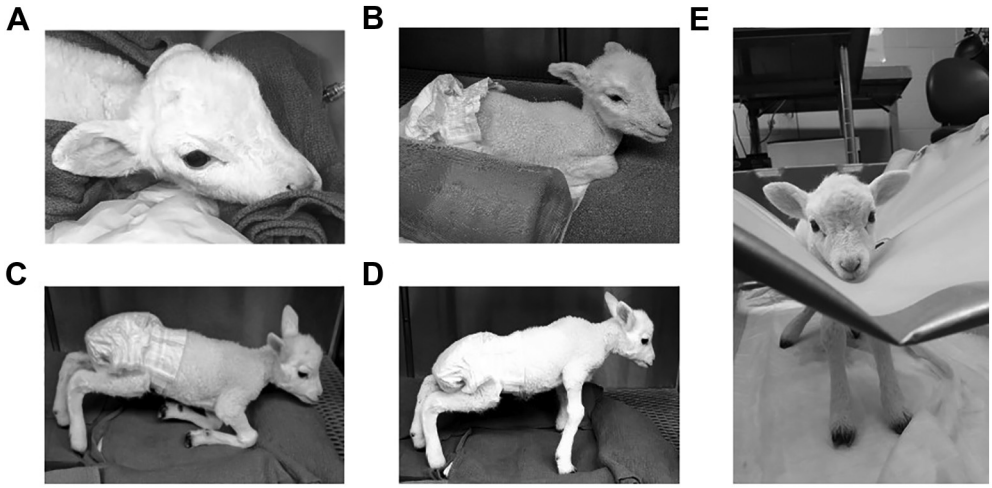

Fig. 6. Representative images of neurologic progression post-extubation. **(A)** Representative lamb prior to developing head support. **(B)** Lamb supporting head at 2 days post-decannulation, followed by **(C)** hind leg support and **(D)** standing at 7 days. **(E)** Lamb within physical therapy sling.

testing for our future experiments. We believe this study is an important step forward in assessing neurologic outcomes on the EXTEND system; we are hopeful that it will someday be possible to prevent much of the major neurologic morbidity and mortality associated with preterm birth.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jpedsurg.2019.12.026.

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