Successful use of an artificial placenta to support extremely preterm ovine fetuses at the border of viability

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[Title] Successful use of an artificial placenta to support extremely preterm ovine fetuses at the border of viability

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[Condensation] Use of an artificial placenta to maintain extremely preterm ovine fetuses (delivery weight ~600-700 g) broadly equivalent to a human fetus at 24 weeks of gestation.
[Short title] Successful treatment of extremely preterm ovine fetuses with ex-vivo uterine environment therapy.

[AJOB at a Glance]

A: To determine the ability of a refined artificial placenta-based life support platform to maintain extremely preterm ovine fetuses 600-700g in weight.

B: The refined artificial placenta platform described herein (reduced priming volume and flow-rates) successfully maintained 7 animals for a period of 120h. Somatic growth and cardiovascular performance were equivalent to in utero controls. There was no identification of infection or inflammation.

C: This study presents the first data, of which we are aware, demonstrating the ability of an artificial placenta-based life support platform to maintain extremely preterm fetuses (600-700g). These data underscore the potential clinical application of the artificial placenta as a treatment option for extremely preterm infants born at the border of viability.

[Key words] Artificial placenta; ex-vivo uterine environment therapy; extremely preterm infants; fetal inflammatory responses; fetal brain injury.
[Abstract]

[Background] Ex-vivo uterine environment (EVE) therapy is an experimental life support platform designed to reduce the risk of morbidity and mortality for extremely preterm infants born at the border of viability (21-24 weeks gestation). To spare the functionally immature lung, this platform performs gas exchange via a membranous oxygenator connected to the umbilical vessels, and the fetus is submerged in a protective bath of artificial amniotic fluid. We and others have demonstrated the feasibility of extended survival with EVE therapy in late preterm fetuses; however, there is presently no evidence to show that the use of such a platform can support extremely preterm fetuses, the eventual translational target for therapy of this nature.

[Objectives] To use our EVE therapy platform to support the healthy maintenance of 600-700 g / 95 d gestational age (equivalent to 24 weeks of human gestation) sheep fetuses. Primary outcome measures were: i) maintenance of key physiological variables; ii) absence of infection; iii) absence of brain injury; and iv) growth and cardiovascular function patterns matching that of non-instrumented, age-matched in utero controls.

[Study Design] Singleton fetuses from eight ewes underwent surgical delivery at 95 d gestation (term=150 d). Fetuses were adapted to EVE therapy and maintained for 120 h with real-time monitoring of key physiological variables. Umbilical artery blood samples were regularly collected to assess blood gas data, differential counts, inflammation and microbial load to exclude infection. Brain injury was evaluated by gross anatomical and histopathological approaches after euthanasia. Nine pregnant control animals were euthanised at 100 d gestation to allow comparative post-mortem analyses. Data were tested for mean differences with ANOVA.
[Results] Seven of eight EVE group fetuses (87.5%) completed 120 h of therapy with key parameters maintained in a normal physiological range. There were no significant inter-group differences (p>0.05) in final weight, crown rump length and body-weight normalized lung and brain weights at euthanasia compared to controls. There were no biologically significant differences in hematological parameters (total or differential leucocyte counts and plasma concentration of TNF-α and MCP-1) (p>0.05). Daily blood cultures were negative for aerobic and anaerobic growth in all EVE animals. There was no difference in airspace consolidation between control and EVE animals, and there was no increase in the number of lung cells staining positive for the T-cell marker CD3. There were no increases in interleukin (IL)-1, 6, 8, TNF-α and MCP-1 mRNA expression in lung tissues compared to the Control group. No cases of intraventricular haemorrhage were observed, and white matter injury was identified only in one EVE fetus.

[Conclusions] For several decades there has been little improvement in outcomes of extremely preterm infants born at the border of viability. In the present study, we report the use of artificial placenta technology to support, for the first time, extremely preterm ovine fetuses (equivalent to 24 weeks of human gestation) in a stable, growth-normal state for 120 h. With additional refinement, the data generated by this study may inform a treatment option to improve outcomes for extremely preterm infants.
[Introduction]

More than one million babies die as a result of prematurity each year worldwide.\(^1\)

Overall, outcomes for preterm infants have markedly improved over the past five decades as a result of improvements in neonatal and obstetric care including antenatal steroid therapy, and the post-natal administration of exogenous surfactant. Despite these significant improvements, infants born at the border of viability (21-24 weeks gestation) remain at significant risk of death, or discharge with one or more severe, life-compromising disabilities.\(^2\) Outcomes of extremely premature (<28 weeks gestation) and extremely low birth weight infants (ELBW <1000 g) have changed little for at least these two decades.\(^2-5\) One of the factors underlying this relative lack of improvement in outcomes is that ventilation-based life support systems may have reached an efficacy threshold that is incompatible with the extremely underdeveloped cardio-pulmonary system seen in this patient demographic.

There are significant limitations in the ability of contemporary neonatal interventions to interface with the extremely preterm cardiopulmonary system. A new therapeutic option, which does not force extremely preterm infants either to be ventilated or to make rapid, precocious changes to their cardiovascular system (i.e. closure of fetal shunts) for pulmonary gas exchange may form the basis of a new therapeutic platform, allowing improved outcomes for extremely preterm infants born at or close to the border of viability.\(^5,6\)

With this objective in mind, we have developed an experimental treatment platform for extremely preterm infants, \textit{ex-vivo} uterine environment (EVE) therapy. The central principle underlying the iterative development of this platform is to treat extremely preterm infants as fetuses, rather than as small babies, and to avoid the use of
pulmonary gas exchange. In previous reports based on our EVE system we 
demonstrated healthy fetal survival for a period of 168 hours (h), and further extended 
that to 336 h in a subsequent set of unpublished experiments.\textsuperscript{7-10} Other investigators 
have reported the successful application of artificial-placenta based therapies for up 
to 669 h with similar concepts and extracorporeal gas exchange systems.\textsuperscript{11-14} 
Accordingly, the feasibility of long survival with a pumpless extracorporeal system has 
been well documented in the experimental literature. 
Despite these successes, the potential clinical utility of artificial placenta-based 
therapies is still far from certain, most notably due to the fact that studies to date have 
employed fetal sheep or goats significantly larger and more mature than the likely 
clinical candidates for artificial placenta therapy. Artificial placenta-based systems 
are expected to be used as a resource for extremely premature fetuses (<100 days 
gestational age (dGA) in lambs or 21-24 weeks of GA in humans). The earliest 
pumpless artificial placenta-based results reported to date have been based on the 
use of a small number of 105-106 dGA fetuses approximately equivalent in size and 
weight to a 29 week GA humans fetus.\textsuperscript{14} Although appropriate from an iterative 
development perspective, if artificial placenta-based systems are to be adopted for 
clinical use, their efficacy must be demonstrated in extremely preterm fetal models. 
This is because the survival rate is significantly and progressively reduced from 28 
weeks of GA and below, with severe disability being significantly more common 
among ELBW compared with very low birth weight (VLBW) infants.\textsuperscript{2,6,15} The aetiology 
is multifactorial but key contributing factors include ventilation of the immature lung, 
which leads to chronic lung injury,\textsuperscript{16-20} immature vascular structure, cardiovascular 
and cerebrovascular autoregulatory systems, which result in higher incidence of one
or more of such disabilities as intraventricular hemorrhage (IVH), white matter injury (WMI) and necrotizing enterocolitis (NEC), and higher rates of life-threatening infection. Rates of cognitive and behavioral challenges remain high with 50% to 60% of extremely preterm infants displaying such disabilities.

With the above in mind, in the present study we report the ability of our refined EVE therapy platform to support clinically relevant, extremely preterm lambs at 95 dGA (24 weeks GA in human equivalent), weighing approximately 600-700 g for a period of 120 h. The primary outcome measures were: i) maintenance of key physiological parameters; ii) absence of infection; iii) absence of brain injury; and iv) growth and cardiovascular function patterns matching that of non-instrumented, age-matched in utero controls.
[Material and Methods]

Experimental Protocol

All procedures were performed in Perth, Western Australia, following review and approval by the Animal Ethics Committee of the University of Western Australia (RA/3/100/1378). Ewes were fasted for 12 h before surgery with ad libitum access to water. Ewes were premedicated, anaesthetized, intubated, and ventilated (acepromazine 0.03 mg/kg and buprenorphine 0.01 mg/kg intramuscularly, midazolam 0.25 mg/kg and ketamine 5 mg/kg intravenously, 1-2% isoflurane in 100% oxygen inhaled, tidal volume 10 mL/kg 8-10 breaths/minute) during the surgical procedure. Intravenous fluids (0.9% NaCl) were administered at a rate of 10 mL/kg/h. The ewe's abdomen was clipped to expose the skin and thoroughly prepared for surgery as described previously.\textsuperscript{7,9,10,37} Ewes in both the EVE and the control groups were euthanized with an intravenous bolus of pentobarbitone (160 mg/kg).

EVE Therapy Platform Refinements

The following refinements were made to the EVE therapy system reported in our previous studies\textsuperscript{7,37}:

i) gas exchange enhanced by lowering the priming circuit volume (from 70 mL to 50 mL) and by the adapting the circuit to use only one high-performance membrane oxygenator;  

ii) using a semi closed-system incorporating a low volume synthetic amniotic fluid bath (reduced from approximately 35 L to 6 L);  

iii) frequent replacement of bath components with UV sterilized synthetic amniotic fluid (every 6 h); and  

iv) discontinuation of carotid artery and jugular vein catheters to reduce the stress on the fetus.

Study Protocol
EVE group: 9 merino-cross ewes with timed, singleton pregnancies were surgically delivered at 95 dGA following daily ultrasound assessment of cardiovascular function.

Surgical delivery

After a maternal laparotomy and hysterotomy, the fetuses were placed inside the sterilized artificial bag, with care taken to ensure umbilical cord patency. Fetuses were intermittently bathed with sterile saline warmed to 40°C. The catheterization procedure was performed prior to delivery as follows, in a procedure taking approximately 10-15 minutes: i) one umbilical artery was catheterized (8 Fr, Bio-Medicus One-piece Femoral Venous Cannulae; Medtronic, Minneapolis, MN) and secured approximately 7-8 cm outside the umbilical ring. The tip of the arterial catheter was sited approximately 2-3 cm from the umbilical ring; ii) one umbilical vein was then quickly cannulated with a 10 Fr custom-made catheter (Nipro Corporation, Osaka, Japan) and secured approximately 1.5-2 cm external to the umbilical ring. The tip of venous catheter was sited approximately 0.5-1 cm past the umbilical ring. The fetuses were then attached to one membranous oxygenator; iii) a second umbilical artery was catheterized while the circuit was temporally circulated with one umbilical artery and umbilical vein and then connected to the circuit; and iv) lastly, the fetus was carefully transferred to the site to be maintained and the bag was promptly filled with synthetic amniotic fluid.

Maintenance after delivery

EVE fetuses were maintained and observed in parallel by a single investigator on a rotating 24 h shift. 24 h after commencement of the EVE therapy, normal intermittent
active fetal swallowing movements, breathing movements, gross fetal body movements, and flexure and extension of limbs were assessed at least every 6 h. The presence of edema, ascites, pleural effusion or bleeding were determined by ultrasound, and by gross examination during necropsy after 120 h of EVE therapy.

Lambs were continuously treated with intravenous heparin (12.5 U/kg/h) to prevent blood coagulation, with the dose adjusted after stabilization in an attempt to maintain an activated clotting time of 180 - 250 s. Prostaglandin E₁ (PGE₁) (40 ng/kg/min, Tandetron; Takata Pharmaceutical, Saitama, Japan) was continuously administered after delivery. Phosphodiesterase type 3-inhibitor (0.5 µg/kg/min, Primacor; Sanofi, Sydney, Australia) was given to assist maintenance of organ blood flow.38 Hydrocortisone (3 mg) was intravenously administered to the fetuses immediately after the induction of the EVE therapy, followed by 6 hourly administrations at a dose of 3 mg (estimated 3-4 mg/kg, ~60 h) and 2 mg (estimated 2-3 mg/kg, ~120 h) respectively to manage fetal critical refractory hypotension which is often observed among extremely premature infants and associated with IVH, PVL and adverse neurodevelopmental outcomes.39-42 Fresh frozen plasma (FFP) was provided for the first hour (10 mL/kg). Midazolam was continuously administered for the first 6 h (0.15 mg/kg/hour).

Intravenous nutrition comprised of glucose (9.5-10%), amino acids (3-3.2 g/kg/day, Pleamin-P injection; Fuso Pharmaceutical, Osaka, Japan), lipid (0.1g/kg/day, intralipid 20%; Fresenius Kabi Australia, Sydney, Australia), vitamin compounds (1/8 vial/day, Daimedin multi inj; Nichi-Iko Pharmaceutical, Tokyo, Japan), micronutrient
(0.1 mL/day, Cizanarine N Inj; Nissin Pharmaceutical, Yamagata, Japan) to provide 70-75 kcal/kg/day.\textsuperscript{14}

To prevent infection, meropenem (15 mg/kg/dose, Ranbaxy; Sydney, Australia) was administered intravenously to the fetuses every 6 h. Intravenous fluconazole (4 mg/kg/dose, Fluconazole-Claris; AFT Pharmaceuticals Pty Ltd, Sydney, Australia) was administered to the fetuses every 24 h. Oxygen supply to the membranous oxygenators was adjusted to maintain fetal $PaO_2$ between 15-30 mmHg.\textsuperscript{43} The fetuses were maintained with EVE therapy for 120 h, followed by euthanasia with intravenous pentobarbitone (160 mg/kg/dose) for measurements of body weight, crown lung length, and tissue sample collection (lung, brain).

**Collection of maternal blood for fetal transfusion**

Meropenem (1 g/dose) was administered to each ewe following the induction of anaesthesia. 100 mL (approximately 2-3% of total circulating blood volume for ewes) was aseptically collected from the jugular vein prior to surgery commencing. Whole blood was immediately heparinized and then used for priming of the artificial placenta circuit.\textsuperscript{44} A further 300-400 mL of whole blood was collected after fetal delivery using a triple-bag blood transfusion system (T331150, Fresenius Kabi, Mount Kuring-gai, Australia). Packed red cells were preserved at 4°C prior to use. Fresh plasma was frozen at -80°C and defrosted on demand.
EVE therapy system components

1) Artificial placenta

The circuit was composed of three main parts: i) outflow tubes; ii) membranous oxygenators; and iii) an inflow tube (all Nipro Corporation, Osaka, Japan). Only one membranous oxygenator per EVE fetus was used. Heparinized polyvinyl chloride tubes were used for both the inflow and outflow tubes. The circuit was primed with 50 mL of heparinized maternal blood. Lipo-PGE\(_1\) was mixed in the circuit priming to prevent umbilical vessel and ductal contraction (100 ng/mL; Sawai Pharmaceutical, Osaka, Japan). The calculated membrane surface area for gas exchange was 0.15 m\(^2\). Extracorporeal pumps were not used to maintain the circuit flow.

2) Amniotic Fluid

Synthetic AF was aseptically prepared as follows: pH: 7.19 ± 0.13, Na+: 116 ± 4 mEq/L, Cl-: 113 ± 5 mEq/L, K+: 6.2 ± 0.8 mEq/L, Ca2+: 1.4 ± 0.3 mEq/L, meropenem: 167 mg/L, fluconazole 3.3 mg/L, all values for pH and electrolytes represent group mean ± standard deviation (SD). The AF was preheated to 39.5-40.0°C and UV-treated for a minimum of 6 h before addition to the AF bath. The AF bath was filled with synthetic AF (6 L) and warmed constantly by two heaters. Heaters were installed at the top (radiant warmer) and at the bottom (contact heat pad) of the AF bath. After the fetus was submerged, AF was maintained at a constant temperature of 38.7 ± 0.3°C (group mean ± SD).

To minimize the risk of microbial colonization, a transparent, sterilized plastic bag was used to contain the AF bath. The sealable single opening of the AF bath was sterilized with 70% ethanol after every opening. The AF bath was rinsed and replaced every 6 h.
after the start of the EVE therapy with 30 L of new synthetic AF which had been UV-treated.

Physiological, haematological, biochemical and microbiological data acquisition

Fetal heart rate and mean arterial pressure were continuously monitored and recorded using a SurgiVet monitor (Smiths Medical, St. Paul, MN). Circuit blood flow (mL/min) was continuously monitored using electromagnetic flow sensors (Transonic 400-Series, Transonic Systems Inc., Ithaca, NY) attached to the arterial positions of the blood circuit, and recorded using a Power-Lab (ADInstruments, Dunedin, New Zealand). Fetal umbilical arterial blood gasses: pH, base excess (BE), pCO₂, pO₂, O₂ saturation (SO₂), O₂ content (CtO₂), sodium ion (Na⁺), potassium ion (K⁺), calcium ion (Ca²⁺), chloride ion (Cl⁻), hemoglobin, lactate and glucose level (Siemens RapidPoint 500, Munich, Germany) and activated clotting times (Haemochron Jr., Accriva Diagnostics, San Diego, CA) were measured at least every 6 h. Fetal umbilical arterial blood samples were collected every 24 h following the induction of the EVE therapy. Haematological analyses, including differential leukocyte counts, biochemical and microbiological analysis (anaerobic and aerobic cultures) were performed by an independent clinical pathology laboratory (Vetpath, Perth, Australia). To prevent hypoxia due to anemia, all sampling was made volume-neutral via the addition of packed red cells and fresh-frozen plasma. Packed red blood cell transfusion generated with maternal blood was performed (10 mL/kg/time) when hemoglobin values fell below 10 g/dL.
Ultrasound Assessment of Cardiac Function

Ultrasound assessments were performed by a single operator before euthanasia. Measurements were conducted with a Philips CX50 system, S5-1 phased array probe (both Philips Healthcare, Netherlands) and associated obstetrics software. For control animal measurements, ewes held in dorsal recumbency and the fetal position from the ventral aspect was confirmed by an operator. The ultrasound beam was focused to obtain a basal four-chamber view, five chamber view, left ventricular outflow tract (LVOT), right ventricular outflow tract (RVOT) view or three vessel (3V) view to check the following items. \(^{45}\) Distance between the attachment point of the mitral valve on the epicardium to the attachment point of the tricuspid valve on the epicardium was measured in four-chamber-view as total cardiac dimension (TCD) (Figure 1A). \(^{46,47}\)

Trans tricuspid and trans mitral inflow were measured using Doppler echocardiography to access the peak early filling (E wave) and late diastolic filling (A wave) velocities in calculation of each E/A ratio (Figure 1B). \(^ {48}\) Cardiac time intervals such as isovolumetric contraction time (ICT), ejection time (ET), isovolumetric relaxation time (IRT) were measured for the left ventricle. Myocardial performance index (MPI) was calculated using the formula defined as MPI = ICT+IRT / ET by Tei and colleagues (Figure 1C). \(^ {49-53}\) The flow velocity wave from the inferior vena cava was recorded in the sagittal view, which included the fetal right atrium, right ventricle and the inferior vena cava. Pulsed Doppler tracings were obtained at the point of the inferior vena cava orifice entering the right atrium. Peak velocity during atrial contraction (A), which frequently has reversed blood velocities away from the heart, and peak velocity during ventricular systole (S) were measured from the recorded
flow velocity waveform of each vein, and the A/S ratio was calculated to obtain the preload index (PLI) (Figure 1D).  

The internal diameter of ductus arteriosus was measured at the confluence of the descending aorta. Color flow Doppler imaging was used to detect the blood flow direction through the ductus arteriosus. Blood flow from the pulmonary artery to the descending aorta was determined as right to left directional flow. These measurements were performed before euthanasia.

**Control group**

After 5 days of ultrasound assessments (as above) nine merino-cross ewes with timed, singleton pregnancies were delivered and euthanized with an intravenous bolus of pentobarbitone (160 mg/kg) at 100±1 dGA to allow comparative measurement of body weight, crown rump length, organ weights (lung, brain), tissue collection (lung, brain), and fetal whole blood and plasma collection at delivery (immediately prior to euthanasia) to perform blood gas analysis, blood corpuscle counts, including differential leukocyte count, biochemical and cytokine analyses. Reliable fetal blood gas data could not be obtained due to euthanasia of ewe and fetus before delivery. Therefore, the blood gas data presented herein were obtained as a reference set from thirteen age-matched (97d GA ± 2 d), null-treatment fetuses previously collected as part of our ovine databank.
Laboratory Analyses

Enzyme-Linked Immunosorbent Assays

Inflammatory protein concentrations for tumor necrosis factor (TNF-α) and monocyte chemoattractant protein 1 (MCP-1) in fetal plasma samples were measured using commercial kits from Kingfisher Biotech (St. Paul, MN), with washing performed on a Biosan plate washer (3D-IW8, Inteliwasher, Biosan, Riga, Latvia) as previously described. Standards (calibration curve R² > 0.99) were assayed in triplicate (average coefficient of variation 7.8%) and samples were assayed in duplicate. The assay limit of detection was <4 pg/mL. 100 μL of each standard or sample was incubated overnight (16 h) at 4°C. Assays were performed in accordance with the manufacturer’s instructions, with absorbance at 450 nm read on a HiPo MPP-96 microplate photometer (Biosan, Riga, Latvia).

Preterm Lung Histology

The right upper lobe of the preterm lung was inflation-fixed in 10% (pH 7.4) neutral buffered formalin with constant pressure (30 cmH2O) for 24 h before being washed in Phosphate Buffered Saline (PBS) and embedded in paraffin. Paraffin sections (5 μm) were stained with Meyer’s hematoxylin and eosin (H&E). Airspace infiltration and consolidation were evaluated by a single investigator blinded to treatment groups. Six fields (200 x total magnification) were assessed for each animal. Indirect immunofluorescent staining of the fetal lung for inflammatory cells expressing CD3 (A0452, Dako, Glostrup, Denmark; working concentration 1:200) was performed as previously published. CD3⁺ cells in fetal lung were quantified by counting positively stained cells in six randomly selected, non-overlapping fields at 200 x total magnification.
magnification. Indirect immunofluorescent staining of positive control (right upper lobe taken from age-matched lambs with significant lung inflammation and airspace consolidation due to *in utero* exposure to *E.coli* lipopolysaccharide) sections, along with primary antibody-only and secondary antibody-only controls, were used to confirm CD3+ staining specificity.

**Preterm Brain Histology**

Perfusion fixed brains with formalin were processed for gross anatomical investigation as described previously. The left-hemisphere was sectioned at 5 mm intervals along the coronal plane from the anterior to posterior surfaces according to the method of Banker and Larroche. Briefly, each 5 mm section was visually inspected for the presence of abnormalities (petechial haemorrhage, focal coagulation necrosis). Every second 5 mm section (four in total) was selected to be embedded in paraffin. 10 µm sections were then stained H&E. Sections were evaluated for the presence of pathological lesions by a single investigator. White matter injury was defined as the presence of focal coagulation necrosis and cellular infiltration localized to within the deep white matter or peripheral to the lateral ventricles. Six non-overlapping fields (40 x total magnification) were assessed for each section.

Indirect immunofluorescent staining of the fetal brain for cells expressing polyclonal oligodendrocyte transcription factor 2 (Oligo2: AB9610, Millipore, Massachusetts, US: working concentration 1:500) and ionized calcium binding adaptor molecule 1 (IBA-1: 01919741, Wako, Virginia, USA: working concentration 1:1000) was performed as previously published. Immunohistochemical outcomes were assessed in 2
sections (anterior basal ganglia level and mammillary body level) per animal and 3 fields of view in periventricular white matter area on non-overlapping sections at 200 x total magnification. Numbers of Oligo2+ and IBA-1+ cells in each field of view were counted manually by single investigator with the results averaged per group and then used for statistical analysis. A single EVE group animal in which periventricular WMI was observed in the H&E section analyses was excluded from the EVE group in group comparison of Oligo2+ and IBA-1+ cells with the Control group. Primary antibody-only and secondary antibody-only controls were used to confirm, Oligo2+ and IBA-1+ staining specificity.

RNA extraction and Quantitative PCR analysis of lung cytokine expression

Total RNA was extracted with Trizol (Life Technologies) from 100 mg of snap-frozen fetal lung (right lower lobe) as previously described. Extracted RNA was treated with Turbo-DNase (Life Technologies) in accordance with manufacturer’s instructions. RNA template was quantified using a Qubit 2.0 fluorometer (Life Technologies) using a broad-range RNA quantitation kit (Life Technologies). RNA extracts were diluted in nuclease-free water (Life Technologies) to a final concentration of 25 ng/µL.

Ovine-specific PCR primers and hydrolysis probes for interleukin (IL)-1β, IL-6, IL-8, TNF-α and MCP-1 (all Life Technologies) were used to perform quantitative PCR. Reactions were performed on a Viia7 thermocycler (Life Technologies) using an EXPRESS One-Step SuperScript qRT-PCR Kit (Life Technologies) with 125 ng of DNA se-treated template fetal tissue RNA in a total volume of 20 µL in accordance with manufacturer’s instructions. Reaction cycling conditions were: 15 min reverse
transcription at 50°C and an initial denaturation/polymerase activation at 95°C for 20 s, followed by 40 cycles of 95°C for 3 s and 60°C for 30 s (data acquisition phase). Target Cq values were normalised to 18S rRNA Cq value and expressed as fold changes relative to pooled control values. Reaction efficiencies were within limits proposed in the MIQE guidelines\textsuperscript{64}. dCq values were used to perform statistical analyses for significant differences between intervention groups vs control group.

**Statistical Analyses**

Statistical analyses were performed using IBM SPSS for Windows, Version 23.0 (IBM Corporation, Armonk, NY). A Chi-Square test was used to test the differences of nominal values between two groups. All numerical data were tested for normality with Shapiro-Wilk tests. Extreme outliers were tested for exclusion with Smirnov-Grubbs tests. In the comparison of two groups, between-group differences in parametric data were tested for significance with *t*-tests, while Mann-Whitney U tests were used for non-parametric data.
[Results]

Physiological variables

Seven of eight fetuses in the EVE Therapy group completed a pre-determined 120 h experimental period. Key physiological data are presented in Figure 2. The adaptation surgery for EVE therapy was not attempted for one fetus due to severe growth restriction (392 g at 95 dGA, making it incompatible with the catheters and circuit design available for this study), and the animal was removed from analyses. Another fetus was euthanized 8 h after the start of EVE therapy due to a critical circuit failure and was also excluded from further analyses.

There were no significant differences in gestational age, sex ratio, fetal body weight, crown rump length, weight corrected crown rump length, lung weight, weight corrected lung weight, brain weight and weight corrected brain weight at the conclusion of the 120 h study between the EVE group and Control group over the experimental period (Table 1).

Cord blood gas data had pO$_2$ values within the target range (15-30 mmHg). Other items; pH, pCO$_2$, BE, SO$_2$, O2, ClO$_2$, Na$^+$, K$^+$, Ca$^{2+}$, Cl$^-$, hemoglobin, lactate level, glucose and activated clotting time also remained clinically acceptable and comparable range to the reference (Table 2).

Statistically, aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGTP), glutamate dehydrogenase (GLDH), total bilirubin, albumin, blood urea nitrogen (BUN) level and BUN/creatinine ratio were
modestly but significantly higher in EVE group than those in the Control group, while there was no difference in creatinine, magnesium (Mg) and phosphorus (P) value at 120h (Table 3).

In terms of ultrasound assessment performed before euthanasia, there was no significant difference in TCD, Tricuspid and Mitral valve E/A ratio, MPI, PLI and dimension of *ductus arteriosus* between the Control group and EVE group. Flow direction of *ductus arteriosus* was from right to left in each of seven EVE animals (Table 4).

Individual case information (2 A-G) is as follows, with data summarized in Table 5:

**Case A-E):** All measured physical variables remained within the respective reference ranges. Normal intermittent active fetal swallowing movements, breathing movements, gross fetal body movements and flexure and extension of limbs were observed throughout the assessed period. There was no edema, ascites, pleural effusion and bleeding. Brain injuries (hemorrhage and WHI) were not detected histopathologically. No bacteria were identified from blood and AF culture bottles.

**Case F):** All measured physical parameters were maintained within the respective reference ranges in first 82 h after adaptation to EVE therapy. Normal intermittent active fetal swallowing movements, breathing movements, gross fetal body movements and flexure and extension of limbs were observed in first 82 h. However, the circuit flow from one umbilical artery deteriorated due to repositioning of catheter
tips in response to active fetal movements and frequent fetal posture change. Then

circuit flow was interrupted for approximately five minutes with an unexpected kink of
the catheters at 82 h after EVE therapy. Circuit flow from one artery was restored with
change of catheter position and angle conducted by an observer. Flow through the
paired artery was not restored to an earlier optimal rate. To prevent further
deterioration in circuit performance fetal movement was reduced via the
administration of midazolam intravenously (0.15 mg/kg/h) until the end of the
experimental period. Although there was no edema, ascites, pleural effusion and
bleeding, review of H&E-stained coronal sections (anterior basal ganglia level)
revealed the presence of focal coagulation necrosis and cellular infiltration consistent
with periventricular leukomalacia. No bacteria were identified from blood and AF
culture bottles.

Case G): All measured physical variables remained within desired reference ranges.

Normal intermittent active fetal swallowing movements, breathing movements, gross
fetal body movements and flexure and extension of limbs were present throughout
the assessed period. However, there was edema, ascites accompanied with bladder
expansion and hydronephrosis. There were no histopathological brain injuries. No
bacteria were identified from the blood culture bottle though *Pseudomonas stutzeri*
was identified in synthetic AF culture bottles taken both from artificial womb and tab at
the end.
EVE Therapy Group Infection and Inflammation

No bacteremia was demonstrated from the blood culture bottles in any of seven EVE animals, though *Pseudomonas stutzeri* was grown from the AF culture bottles in one EVE animal at the end of the experimental period. There were no significant differences in the numbers of WBC, neutrophils, lymphocytes or monocytes between the Control group and the EVE group (Figure 3 A) at the conclusion of the experimental period. Fetal plasma TNF-α protein concentrations were low and equivalent between Control and EVE group animals. There was no significant difference in MCP-1 protein between the Control group and the EVE group (70.7 [47.7-95.5] pg/mL vs 42.4 [27.4-86.7] pg/mL, *p*=0.351, expressed as median [interquartile range]. There were no significant differences in the relative mRNA expression of IL-1β, 6, 8, TNF-α and MCP-1 were identified in lung samples taken from EVE Therapy animals, compare to Control animals (Figure 3 B). No significant infiltrations of cells were identified in the pulmonary air spaces in any of seven EVE animals that completed their protocol (Figure 4). Immunofluorescent staining for pulmonary CD3+ cells was unremarkable, with no differences detected between Control and Eve Therapy samples (data not shown).

Brain histopathology

One animal (Case F) had WMI in H&E-stained coronal sections (anterior basal ganglia level). There was no evidence of haemorrhage in any of seven EVE animals, or of WMI in the remaining six EVE animals in gross anatomical and H&E stained histopathological analyses of coronal brain sections. There was no significant difference in the number of either Oligo2+ or IBA-1+ cells in periventricular white
matter between the Control group and the EVE group, apart from one EVE animal with periventricular WMI (Case F) (Figure 5).
The primary finding of this study is that EVE Therapy allowed a 120 h period of survival in a group of seven extremely preterm lambs (Figure 2, Table 6). To our knowledge this is the first report of an artificial placenta-based life support system being used to sustain extremely preterm fetuses (600-700 g) approximating the size and weight of a human fetus close to the border of viability (21-24 weeks of gestation). Although there was one case of circuit failure in our study, animals had otherwise stable maintenance from a haemodynamic viewpoint (Figure 2, Table 1-3,5) without any difference in cardiac ultrasound parameters between the Control group and EVE group (Table 4). All animals were free of bacteraemia and no systemic inflammatory changes were detected (Figures 3, 4 Table 5, 6). Although a treatment time of 120 h may be limited in terms of clinical use, the data from this study shows the potential of EVE therapy as a clinical platform for extremely preterm infants.

**EVE Therapy Refinements**

Several refinements were made to the EVE therapy system used in our previous studies.7,37 Firstly, a reduced circuit priming volume was achieved without conveying adverse effects on oxygenation, circulation or cardiac performance. Further improvement may be required for more premature infants.

Secondly, the volume of synthetic amniotic fluid bath was reduced from approximately 35 L to 6 L. In addition, a continuous AF circulation system equipped with an extracorporeal UV filter and particle filter was removed to reduce the extra priming volume for AF circulation and the capacity for biofilm formation.
Finally, catheters were not placed into the carotid artery and jugular vein, reducing the impost on the fetus. This is important because the preterm skin is extremely fragile and the surgical wound site may act as an infection portal.

**Maintenance of key physiological parameters**

Key physiological parameters and blood lactate levels remained within their reference ranges, or rapidly returned to reference range after EVE therapy was started (Figure 2), though total lung flow volume was maintained within or partially above cited normal range (150-250 mL/kg/min).\(^{65-67}\) Regardless of the total lung flow being partially maintained above the cited normal range, this deviation did not seem to correlate with clinically significant effects on fetal circulation, based on fetal lactate levels and ultrasound data. Thus, the ostensibly elevated flow observed in this study may be appropriate for extremely preterm fetuses being maintained on our system. It was demonstrated that EVE group animals were equivalent to the Control group animals in fetal weight, crown-rump length, lung and brain weights. Thus, 120 h of EVE maintenance did not cause obvious growth restriction compared to the control animals, although a treatment time of 120 h may be too short to examine fetal growth adequately (Table 1).

**Ultrasound data**

The main aim of these ultrasound measurements was the assessment of cardiac dysfunction in EVE therapy animals. Generally, TCD was measured as an index for cardiac size because cardiac size is relative to cardiac dysfunction.\(^{46,47}\) The EVE group animals were equivalent to the Control group animals in TCD. Regarding E/A
ratio, the majority of blood passing to the ventricle is reportedly propelled by atrial contraction, and the E/A ratio is usually below 1 for fetuses.\textsuperscript{68} Although, in adults, decreased values are considered a sign of diastolic dysfunction, fetuses with heart failure have been reported to have increased E/A ratios.\textsuperscript{69} For cardiac dysfunction accompanied with intrauterine growth restriction, an increase E/A ratio is likely to be observed.\textsuperscript{70-72} In this study, E/A ratio both of the Control animals and the EVE animals was below 1 and the EVE animals were equivalent to the Control group animals in both tricuspid valve and mitral valve E/A ratio. As part of a fetal evaluation, MPI is considered as a reliable early marker of fetal cardiac dysfunction. It likely represents initial stages of cardiac adaptation to different perinatal insults.\textsuperscript{50-52} MPI data from EVE group animals were equivalent to the Control group animals and both within similar ranges (0.28-0.44) to human data.\textsuperscript{53} There is also an association between changes in circulation and changes in the IVC flow pattern. Increased reverse flow in the IVC during atrial contraction has been reported to suggest cardiac dysfunction.\textsuperscript{73,74} Thus, PLI was adapted for use in this study. The EVE Therapy Group animals were equivalent to the Control group animals in PLI. As for fetal circulation, ductus arteriosus was kept open and fetal circulation (R→L direction through ductus arteriosus) was maintained with EVE therapy over time 120 h experimental period.

**Brain injury**

Gross anatomical observation and assessment of H&E stained brain sections identified the presence of pathological lesions in only one of seven EVE group animals (Case F). In Case F, periventricular WMI was identified in one field at the level of the anterior basal ganglia. WMI is the most common brain damage in preterm
infants, born < 32 weeks GA, typically localized to the periventricular white matter area in a diffuse or focal pattern.\textsuperscript{75} Thus, further analysis for ischemic change at periventricular area was conducted to compare remained EVE animals in which periventricular WMI were not identified by H&E stain with the Control animals. Reportedly Oligo2+ cells are likely to decrease in WMI, thus we used Olig2 to identify oligodendrocyte lineage cells,\textsuperscript{60,76} and did not characterize the maturational progression of oligodendrocytes in the current study. Microglial activation is one of the first steps in the process of neuroinflammation, and in turn is a critical contributor towards WMI. Therefore, activated microglia are likely to increase in WMI and IBA-1 was used to identify activated microglia as described previously.\textsuperscript{60,77,78} However, the result of immunostaining demonstrated that there was no significant difference in the number of Oligo2+ and IBA-1+ cells between the Control group animals and EVE group animals which were not identified WMI in H&E stain investigation. Although the cause of this injury is unclear, it might derive from acute hypoxic-ischemic injury resulting from occlusion of the catheters accompanied with complete interruption of the circuit flow at 82 h into the experiment, and transient elevation of lactate prior to resumption of the circuit flow. Therefore, further improvements to the stability of the EVE circuit will be a key to reduce WMI risk for extremely premature infants.

\textit{Success rate}

The survival rate with EVE therapy was 87.5%. Although this period may seem somewhat brief for an evaluation of survival rate, approximately 50% of deaths among infants <28 weeks occurs in first 72 h (mortality of <28 weeks GA is overall approximately 30% of total deliveries).\textsuperscript{6} Thus, survival rate over the first 120 h of
ex-utero life may provide a useful guide as to the potential clinical significance of EVE therapy.

**Limitations and challenges to be overcome**

The primary limitation of this study is the small sample number and short trial period. The management of preterm lambs with our EVE Therapy system requires a substantial amount of infrastructure and constant monitoring, which limits the sample number achievable. Furthermore, as this was the first attempt to adapt extremely early gestational fetuses to an artificial placenta system, we elected to limit its duration of the experiment to 5 days, allowing us to establish the acute efficacy of our system and, as a result, engineer additional refinements necessary to support a longer-term study. In our previous studies, the critical issues which affected fetal survival and wellbeing (including those associated with brain injury and infection) occurred in the acute phase of the study, within the first 120 h.

Although we have assessed a range of key physiological and haematological variables in determining fetal well-being over the 120 h experiments, we have not performed granular assessments of fetal growth and organ development. Extensive assessments of this nature, in particular lung and brain development including differentiation or gain of volume and respective function, will be a key element of future studies; such analyses will be allowed by well-planed large-scale long studies using our refined EVE Therapy system, and will be essential to demonstrate the capacity for clinical utility of this concept for extremely preterm infants.
An additional limitation of importance is uncertain hydronephrosis with bladder expansion (Figure 2G, Table 5). An obvious cause such as obstruction could not be clarified at the necropsy. Although elimination half-life of midazolam causes urinary retention in preterm infants, the active movement observed by the fetuses after midazolam cessation (6 h) may indicate side effects of the midazolam, such as urinary retention, are unlikely. Accidental ligation of urachus when umbilical vessels were tied off during catheterisation might cause expansion of the bladder and hydronephrosis. This may represent an animal limitation and warrants further investigation as this model continues to be developed.

There are also a number of technical challenges to be overcome before the clinical utility of EVE therapy can be tested. In the present study, the two cases of adverse outcomes (one fetal death and one incidence of WMI) related to issues with catheter placement and occlusion. At present, we use generic catheters with sizing decided a priori. A bespoke catheter system based on a less rigid and shorter arm catheter may allow for improved circuit performance. Furthermore, for clinical application a range of catheter sizes and lengths will likely be required to accommodate inter-patient variability in umbilical cord size, length and perhaps disease status (i.e. funisitis).

In the present study we administered hydrocortisone to manage refractory hypotension due to preliminary findings that untreated fetuses not be maintained within normal physiological variables for more than 24 h after induction of the EVE therapy due to refractory (to continuous volume load; 20 mL/kg/h and inotropic drug; dopamine 10 mcg/kg/min) hypotension, eventually resulting in circuit failure and fetal
death. Hypotension is reported to occur in approximately 20-45% of the preterm infants\textsuperscript{82,83} and its prevalence is inversely related to GA.\textsuperscript{84} About 25% of the ELBW with hypotension does not respond to the treatment with either volume and inotropic drug, and requires hydrocortisone, independent of serum cortisol concentrations, to normalize blood pressure.\textsuperscript{40,85,86} Furthermore, it is described that hypotension in ELBW infants who are <26 weeks of GA and unexposed to antenatal steroid are likely to have refractory hypotension, and an initial therapy with, or earlier institution of hydrocortisone is beneficial to such neonates.\textsuperscript{39} Our hydrocortisone maintenance assisted us in achieving 120 h survival time for seven in eight EVE animals. However, adverse effect for the growth or neurodevelopment given by the use of hydrocortisone and appropriate dose of hydrocortisone for hypotension is still unoptimized.\textsuperscript{87-89} Evaluation of long-term effects on the fetus due to hydrocortisone treatment is also likely an important element of future work in this space.

An additional matter of importance is that EVE group animals had significant increases of AST, ALT, GGTP and LDH compared to the Control animals, which generally indicates liver dysfunction, though not clinically severe. EVE group animals also had BUN increase without significant creatinine increase compared to the Control group (Table 3). One potential cause is an excessive administration of amino acids, although the precise cause is still uncertain and several factors such as drug given to fetuses and parental nutrition itself\textsuperscript{90} have to be taken into consideration as the cause. Because it’s accompanied with increase of BUN, BUN/creatinine ratio and plasma albumin level without creatinine increase or obvious episode of fetal bleeding. Dietary protein overload induces liver disfunction involved with the increase of BUN,
BUN/creatinine ratio and plasma albumin level, resulting in increased likelihood of renal dysfunction in premature babies.\textsuperscript{91,92} Although modest doses of amino acids (3 g for total 70-75 kcal/kg for 95 dGA) for 120 h were administered in this study, compared to previously described dose (6 g for total 70 kcal for 105-111 dGA) for two to four weeks which did not cause any elevation of fetal plasma AST (27U/L), ALT(3 U/L) and BUN (24 mg/dl),\textsuperscript{14} this dose of amino acids might be an overload for extremely premature fetuses. One solution may be to increase the total calorific intake to balance amino acid metabolism, though appropriate administration of amino acids and total energy is still controversial, even in the human clinical field.\textsuperscript{92}

Continuous administration of high-caloric nutrition causes hyperglycemia, however, which requires intravenous insulin administration to be administered as described previously.\textsuperscript{14} It may generate another risk as hypoglycemia, which is associated with brain disorder and may give a diabetes-like influence to the fetus during longer studies, while it can enhance protein synthesis and growth.\textsuperscript{93,94} Pulsatile administration of high-caloric nutrition may offer a solution to this problem.\textsuperscript{95} On the other hand, reducing amino acid dose may cause growth restriction.\textsuperscript{92} Thus, we are still in the process of determining appropriate nutrition. The effect of long-time parental nutrition itself on extremely premature fetal liver also requires further assessment in future studies.
[Conclusions]

Using extremely preterm sheep fetuses, we report a 120 h period of well-controlled survival and control-equivalent growth using our EVE therapy system, based on the use of an artificial placenta for gas exchange. Extremely preterm sheep fetuses were observed to be free of infection and systemic inflammation. Improving the survival rate, and stabilizing circuit performance to protect against white matter injury will be an important focus of future research. Although still preliminary, these novel findings demonstrate the potential clinical utility of a further refined EVE therapy system to improve outcomes for extremely preterm infants at the border of viability.
[Acknowledgements]

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[References]


49. Tei C, Ling LH, Hodge DO, et al. New index of combined systolic and diastolic myocardial performance: a simple and reproducible measure of cardiac...


<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>EVE group</th>
<th>Statistical test</th>
<th>p value</th>
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<td>Number</td>
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<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age at induction of EVE therapy (d)</td>
<td>-</td>
<td>94.9 ± 0.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gestational age at conclusion (d)</td>
<td>99.8 ± 0.7</td>
<td>99.9 ± 0.7</td>
<td>Mann-Whitney U</td>
<td>0.812</td>
</tr>
<tr>
<td>Sex (male/female)</td>
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<td>2/5</td>
<td>Chi-square test</td>
<td>0.286</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>1076 ± 72</td>
<td>1051 ± 127</td>
<td>t-test</td>
<td>0.62</td>
</tr>
<tr>
<td>Crown rump length (cm)</td>
<td>32.7 ± 1.1</td>
<td>32.1 ± 2.3</td>
<td>t-test</td>
<td>0.525</td>
</tr>
<tr>
<td>Weight corrected crown rump length (cm/kg)</td>
<td>30.5 ± 1.1</td>
<td>30.7 ± 1.6</td>
<td>t-test</td>
<td>0.681</td>
</tr>
<tr>
<td>Lung weight (g)</td>
<td>39.8 ± 3.2</td>
<td>39.3 ± 2.9</td>
<td>t-test</td>
<td>0.713</td>
</tr>
<tr>
<td>Weight corrected lung weight (g/kg)</td>
<td>37.0 ± 2.2</td>
<td>37.6 ± 3.3</td>
<td>t-test</td>
<td>0.673</td>
</tr>
<tr>
<td>Brain weight (g)</td>
<td>27.3 ± 1.8</td>
<td>26.6 ± 1.7</td>
<td>t-test</td>
<td>0.397</td>
</tr>
<tr>
<td>Weight corrected brain weight (g/kg)</td>
<td>25.4 ± 1.3</td>
<td>25.4 ± 1.7</td>
<td>Mann-Whitney U</td>
<td>0.266</td>
</tr>
</tbody>
</table>

**Table 1. Comparison of fetal data at necropsy.** Values are expressed as the group mean ± SD. *p*<0.05 is considered as significant difference vs. value for the Control group.
Table 2. Blood gas data throughout experiments in the EVE group.

Seven EVE animals were analyzed throughout the experiment. Umbilical arterial blood was collected for blood gas data every 6 h after the start of the EVE therapy. Values are expressed as the group mean ± SD. SO₂, O₂ saturation; CtO₂, O₂ content = hemoglobin (g/dl) × 1.34 × SpO₂ (%) / 100 + pO₂ × 0.003; Na⁺, sodium ion; K⁺, potassium ion; Ca²⁺, calcium ion; Cl⁻, chloride ion. The reference data were obtained from thirteen age-matched (97±2 dGA), null-treatment fetuses, which were previously collected for our ovine databank.

<table>
<thead>
<tr>
<th></th>
<th>Reference</th>
<th>EVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.38 ± 0.03</td>
<td>7.39 ± 0.05</td>
</tr>
<tr>
<td>pCO₂ (Torr)</td>
<td>42.4 ± 2.9</td>
<td>41.7 ± 3.3</td>
</tr>
<tr>
<td>pO₂ (Torr)</td>
<td>25.1 ± 2.1</td>
<td>24.6 ± 3.6</td>
</tr>
<tr>
<td>Base excess (mmol/L)</td>
<td>-0.2 ± 1.8</td>
<td>-0.3 ± 3.5</td>
</tr>
<tr>
<td>SO₂ (%)</td>
<td>65.5 ± 5.9</td>
<td>55.6 ± 10.0</td>
</tr>
<tr>
<td>CtO₂ (mL/dL)</td>
<td>8.9 ± 1.2</td>
<td>8.2 ± 1.7</td>
</tr>
<tr>
<td>Hemoglobin (g/L)</td>
<td>93 ± 9</td>
<td>109 ± 14</td>
</tr>
<tr>
<td>Lactate level (mmol/L)</td>
<td>1.7 ± 0.4</td>
<td>1.2 ± 0.6</td>
</tr>
<tr>
<td>Na⁺ (mmol/L)</td>
<td>134 ± 3.1</td>
<td>138 ± 6.5</td>
</tr>
<tr>
<td>K⁺ (mmol/L)</td>
<td>4.0 ± 0.3</td>
<td>4.2 ± 0.8</td>
</tr>
<tr>
<td>Ca²⁺ (mmol/L)</td>
<td>1.4 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Cl⁻ (mmol/L)</td>
<td>103 ± 1.7</td>
<td>107 ± 7.5</td>
</tr>
<tr>
<td>Glucose (mmol/L)</td>
<td>1.4 ± 0.2</td>
<td>2.1 ± 1.5</td>
</tr>
<tr>
<td>Activated clotting time (s)</td>
<td>-</td>
<td>221± 32</td>
</tr>
<tr>
<td></td>
<td>Control group</td>
<td>EVE group</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------</td>
<td>-----------</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>27 ± 6</td>
<td>68 ± 35 *</td>
</tr>
<tr>
<td>ALT (U/L)</td>
<td>3 ± 3</td>
<td>17 ± 9 *</td>
</tr>
<tr>
<td>GGTP (U/L)</td>
<td>11 ± 2</td>
<td>34 ± 20 *</td>
</tr>
<tr>
<td>GLDH (U/L)</td>
<td>4 ± 2</td>
<td>25 ± 22 *</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>0.7 ± 0.2</td>
<td>2.3 ± 1.2 *</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>1.7± 0.1</td>
<td>1.9 ± 0.1*</td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>19.0 ± 3.6</td>
<td>39.4 ± 21.7*</td>
</tr>
<tr>
<td>Creatinine (mg/d/L)</td>
<td>0.91 ± 0.11</td>
<td>0.88 ± 0.40</td>
</tr>
<tr>
<td>BUN/Creatinine ratio</td>
<td>21.7 ± 5.9</td>
<td>44.4 ± 5.6 *</td>
</tr>
<tr>
<td>Mg (mmol/L)</td>
<td>1.2± 0.2</td>
<td>1.1 ± 0.1</td>
</tr>
<tr>
<td>P (mmol/L)</td>
<td>2.2± 0.3</td>
<td>2.0 ± 0.2</td>
</tr>
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</table>

Table 3. Comparison of fetal chemical data at 120h. Nine Control animals and seven EVE animals were analyzed. Respective values are expressed as the group mean ± SD. A t-test was conducted for statistical analysis. Significant difference vs. value for the Control group is indicated: * , p<0.05 and [95% confidence interval]. AST, aspartate aminotransferase; ALT, alanine aminotransferase, GGTP, gamma-glutamyl transpeptidase; GLDH, glutamate dehydrogenase; BUN, blood urea nitrogen; Mg, magnesium; P, phosphates.
<table>
<thead>
<tr>
<th></th>
<th>Control group</th>
<th>EVE group</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cardiac dimension (mm)</td>
<td>24.1 ± 0.9</td>
<td>25.3 ± 1.2</td>
<td>0.081</td>
</tr>
<tr>
<td>Tricuspid valve E/A ratio</td>
<td>0.75 ± 0.12</td>
<td>0.69 ± 0.06</td>
<td>0.228</td>
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<tr>
<td>Mitral valve E/A ratio</td>
<td>0.76 ± 0.09</td>
<td>0.69 ± 0.05</td>
<td>0.105</td>
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<tr>
<td>Myocardial performance index</td>
<td>0.39 ± 0.08</td>
<td>0.43 ± 0.06</td>
<td>0.264</td>
</tr>
<tr>
<td>Preload index</td>
<td>0.32 ± 0.09</td>
<td>0.36 ± 0.11</td>
<td>0.485</td>
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<tr>
<td>Dimension of ductus arteriosus (mm)</td>
<td>5.2 ± 0.4</td>
<td>4.3 ± 0.8</td>
<td>0.099</td>
</tr>
<tr>
<td>Direction of ductus arteriosus flow</td>
<td>Right → Left</td>
<td>Right → Left</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 4. Comparison of fetal cardiac ultrasound data at the conclusion. Nine Control animals and seven EVE animals were analyzed. Values are expressed as the group mean ± SD. *t*-test was conducted for statistical analysis. *p*<0.05 was considered as significant difference. Blood flow from the pulmonary artery to the descending aorta was determined as right to left directional flow.
### Table 5. Case summary of EVE Therapy group animals.

<table>
<thead>
<tr>
<th>Case</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>G</th>
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<tr>
<td>Swallowing movement</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(24-82H) +</td>
<td>+</td>
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<tr>
<td>Breathing movement</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(24-82H) +</td>
<td>+</td>
</tr>
<tr>
<td>Gross body movements</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(24-82H) +</td>
<td>+</td>
</tr>
<tr>
<td>Flexure and extension of limbs</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>(24-82H) +</td>
<td>+</td>
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<tr>
<td>Edema</td>
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<td>Bleeding</td>
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<tr>
<td>Culture from amniotic fluid in artificial uterus</td>
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<td>Pseudomonas stutzeri</td>
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<td>Circuit trouble due to a kink of the tip of the catheters</td>
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</tbody>
</table>

Normal intermittent active fetal swallowing movement, breathing movements, gross fetal body movements, and flexure and extension of limbs were assessed at least every 6 h. Edema, ascites, pleural effusion, bleeding and another specific issue were identified during necropsy after 120 h of EVE Therapy.
White matter injury was identified in H&E-stained coronal sections. Samples for the culture bottles were collected from fetal umbilical artery, synthetic AF in artificial womb and sterilized tab at the end of the experimental period.
Table 6. Success rate over time in the EVE group. Infection was determined by microbial culture, differential cell counts, lung histopathology. Brain injury was determined by the presence of IVH, WMI identified in gross anatomical observation and historical assessment of the brain sections.
[Figure legends]

**Figure 1. Representative images of the cardiac ultrasound variables.**

Figure 1 A; the arrow indicates distance between the attachment point of the mitral valve on the epicardium to the attachment point of the tricuspid valve on the epicardium as total cardiac dimension (TCD). Figure 1B; atrioventricular flow was measured using Doppler echocardiography to assess the $E/A$ ratio. The arrow with character $E$ indicates the peak early filling ($E$ wave) velocity and another arrow with character $A$ indicates the late diastolic filling ($A$ wave) velocity. Figure 1 C; the arrow with character $a$ indicates isovolumetric contraction time. The arrow with character $b$ indicates ejection time. The arrow with character $c$ indicates isovolumetric relaxation time. Myocardiac performance index (MPI) was calculated as $MPI = a + c / b$.

Figure 1D; the flow velocity wave from the inferior vena cava was recorded using Doppler echocardiography. The arrow with character $A$ indicates the peak velocity during atrial contraction and the arrow with character $S$ indicates the peak velocity during ventricular systole. $A/S$ ratio was calculated to obtain the preload index.

**Figure 2. Changes in fetal physiological and biochemical variables over time in the EVE group.**

The horizontal axis represents the time after the induction of the EVE therapy (h). The black solid lines show total oxygenator (circuit) blood flow (mL/kg/min); the gray solid lines show HR, heart rate (beats/min); the gray dotted lines show MAP, mean arterial pressure (mmHg); The black closed triangles show $SO_2$, arterial oxygen saturation (%); the black closed circles show $pCO_2$ (Torr); the black closed squares show Lac, blood lactate level (mmol/L). Only the blood lactate levels use the right scale bar.

**Figure 3. Differential Cell Counts (blood) and Cytokine mRNA Expression (lung)**
In panel A; WBC, white blood corpuscle. Nine Control animals and seven EVE animals were analyzed. All values are presented as bar charts with the group mean and with whiskers representing SD. The white bars indicate the Control group. The gray bars indicate the EVE group. Differences of values between the groups were tested for significance using \( t \)-test with \( p \) value <0.05 accepted as significant.

In panel B; Nine control animals and seven EVE animals were analyzed. Relative fold changes in cytokine (Interleukins-1\( \beta \), 6, 8, tumour necrosis factor \( \alpha \) and monocyte chemoattractant protein 1) mRNA expression between Control group and EVE group in lung tissue samples. Star indicates outliers. All values are presented as box plots with the group median and with whiskers representing maximum and minimum values. White box indicates the Control group and gray box indicates EVE group. Respective differences of values were tested for significance using Mann Whitney U test with \( p \) value <0.05 accepted as significant.

**Figure 4. Fetal lung inflammation**

For histological assessments, 6 fields were assessed for each animal (\( n=9 \)/the Control group, \( n=7 \)/the EVE group). Panels A, C, Control tissue. Panels B, D, EVE therapy group. Images are representative of: H&E staining of fetal lung visualized at 100 x (Panel A, B) and 200 x (Panel C, D) total magnification. All scale bars indicate 100 \( \mu \)m.

**Figure 5. Representative images of brain histology.**

Seven EVE animals (Cases A-G) were analyzed with H&E stain. 6 fields from four 5mm serial sections were assessed for each animal. Panel A, representative gross appearance of coronal section. Representative image of white matter injury from Case F identified in investigation with H&E staining showing necrosis and cellular
infiltration. Images are inspected at 100 x total magnification (Panel B) and 200 x (Panel C); scale bar represents 100 μm. Nine Control animals and Six EVE animals (Cases A-E, G) without periventricular WMI identified by H&E staining were analyzed for immunofluorescent staining (Oligo2 and IBA-1). 3 fields in periventricular area from two 5mm serial sections were assessed for each animal. Representative images of Oligo2+ cells from Control group (Panel D) and EVE group (Panel E). Representative images of IBA-1+ cells from Control group (Panel G) and EVE group (Panel H). Images are inspected at 200 x total magnification. Comparison of the number of Oligo2+ (Panel F) and IBA-1; cells (Panel I) between Control group and EVE group without WMI identified in H&E investigation. All values are presented as box plots with the group median and with whiskers representing maximum and minimum values. White box indicates the Control group and gray box indicates EVE group. Respective differences of values were tested for significance using Mann Whitney U test with p value <0.05 accepted as significant.