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Successful use of an artificial placenta-based life support system to treat extremely preterm ovine fetuses compromised by intrauterine inflammation.

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[Title] Successful use of an artificial placenta-based life support system to treat extremely preterm ovine fetuses compromised by intrauterine inflammation.

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[Condensation] Use of an artificial placenta to maintain extremely preterm ovine fetuses (delivery weight ~600-700 g) compromised by intrauterine inflammation.

[Short title] Successful treatment of extremely preterm ovine fetuses exposed to intrauterine inflammation with ex-vivo uterine environment therapy.

[AJOG at a Glance]

A: To determine the ability of a refined artificial placenta-based life support platform to maintain extremely preterm ovine fetuses exposed to intrauterine inflammation, a common antecedent of extremely early preterm birth.

B: Our artificial placenta platform was successfully used to maintain eight extremely preterm fetuses for a pre-determined period of 120h. Biological parameters and cardiovascular performance were largely equivalent to *in utero* controls. There was no identification of infection or additional injury.

C: This study presents the first data, of which we are aware, demonstrating the ability of an artificial placenta-based life support platform to support extremely preterm ovine fetuses compromised by exposure to intrauterine inflammation. Intrauterine inflammation is a common antecedent of extremely early preterm birth and is associated with adverse outcomes. These data underscore the potential clinical application, and highlight the additional refinements required before an artificial placenta-based system can be considered a viable 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 response; chorioamnionitis; fetal brain injury.

[Glossary]

Artificial placenta: a gas exchange device comprised of one or more membranous oxygenators connected to the umbilical vessels by in-dwelling catheters. Circuit pressure is provided by the fetal heart.

***Ex-vivo* uterine environment (EVE) therapy:** the use of an artificial placenta to provide life support to an extremely preterm infant by simulating placental gas exchange.

Extremely preterm infants: infants born close to the border of viability i.e. 21-24 weeks' gestation.

Glucocorticoids: synthetic steroid hormones that exert pleiotropic signaling activity via activation of the glucocorticoid receptor. Widely used in obstetrics to precociously mature the preterm lung in anticipation of preterm delivery.

Lipopolysaccharides: large lipid and polysaccharide molecules found in the outer layer of Gram-negative bacteria. Lipopolysaccharides elicit a robust inflammatory response that can be used to simulate intrauterine inflammation and generate chorioamnionitis.

[Abstract]

[Introduction] *Ex-vivo* uterine environment (EVE) therapy is an experimental intensive care strategy for extremely preterm infants born between 21 and 24 weeks of gestation. Gas exchange is performed by membranous oxygenators connected by catheters to the umbilical vessels. The fetus is submerged in a bath of synthetic amniotic fluid. The lungs remain fluid-filled and pulmonary respiration does not occur. Intrauterine inflammation is strongly associated with extremely early preterm birth and fetal injury. Presently, there are no data of which we are aware to show that artificial placenta-based systems can be used to support extremely preterm fetuses compromised by exposure to intrauterine inflammation.

[Objectives] To evaluate the ability of our EVE therapy platform to support extremely preterm ovine fetuses (95 d gestational age; approximately equivalent to 24 weeks of human gestation) exposed to intrauterine inflammation for a period of 120 hours, the following primary endpoints were chosen: **i)** maintenance of key physiological variables within normal ranges; **ii)** absence of infection and inflammation; **iii)** absence of brain injury; and **iv)** gross fetal growth and cardiovascular function matching that of age-matched *in utero* controls.

[Study Design] Ten ewes with singleton pregnancies were each given a single intraamniotic injection of 10 mg *E.coli* lipopolysaccharides (LPS) under ultrasound guidance 48h before undergoing surgical delivery for adaptation to EVE therapy at 95d gestation (term=150d). Fetuses were adapted to EVE therapy and maintained for 120h with constant monitoring of key vital parameters (EVE Group) before being euthanised at 100d equivalent gestational age. Umbilical artery blood samples were regularly collected to assess blood gas data, differential counts, biochemical

parameters, inflammatory markers and microbial load to exclude infection. Ultrasound was conducted at 48 h after intraamniotic LPS (before surgery) to confirm fetal viability and at the conclusion of the experiments (before euthanasia) to evaluate cardiac function. Brain injury was evaluated by gross anatomical and histopathological investigations. Eight singleton pregnant control animals were similarly exposed to intraamniotic LPS at 93d gestation and were euthanized at 100 d gestation to allow comparative post-mortem analyses (Control Group). Bio-banked samples from age-matched saline-treated animals served as an additional comparison group. Successful instillation of LPS into the amniotic fluid exposure was confirmed by amniotic fluid analysis at the time of administration, and by analyzing cytokine levels in fetal plasma and amniotic fluid. Data were tested for mean differences with ANOVA.

[Results] Six out of eight LPS Control Group (75%) and eight out of ten EVE Group fetuses (80%) successfully completed their protocols. Six of eight EVE Group fetuses required dexamethasone phosphate treatment to manage profound refractory hypotension. Weight and crown rump length was reduced in EVE Group fetuses at euthanasia compared to LPS Control Group fetuses ($p < 0.05$). There were no biologically significant differences in cardiac ultrasound measurement, differential leucocyte counts ($p > 0.05$), plasma tumour necrosis factor α (TNF- α), monocyte chemoattractant protein-1 (MCP-1) concentrations ($p > 0.05$), or liver function tests between groups. Daily blood cultures were negative for aerobic and anaerobic growth in all EVE Group animals. No cases of intraventricular haemorrhage were observed. White matter injury was identified in three of six LPS Control Group fetuses and three of eight EVE Group fetuses.

1 **[Conclusions]** We report the use of an artificial placenta-based system to support
2 extremely preterm lambs compromised by exposure to intrauterine inflammation. Our
3 data highlight key challenges (refractory hypotension, growth restriction and white
4 matter injury) to be overcome in the development and use of artificial placenta
5 technology for extremely early preterm infants. As such challenges appear largely
6 absent from studies based on healthy pregnancies, additional experiments of this
7 nature using clinically relevant model systems are essential for further development
8 of this technology and its eventual clinical application.

[Introduction]

Preterm birth (delivery before 37 weeks' gestation) is a leading cause of death under 5 years and of long-term disability.¹ More than one million premature babies die each year worldwide.² Overall outcomes for preterm infants have markedly improved over the past five decades as a result of advances in neonatal and obstetric care including antenatal steroid therapy, and the post-natal administration of exogenous surfactant.³

Infants born at the border of viability (21-24 weeks gestation) continue to have an extremely high risk of death, or severe complications.¹ Outcomes for extremely premature (<28 weeks gestation) and extremely low birth weight infants (ELBW <1000 g) have improved little over the past two decades.^{1,4-6} One of the factors responsible for this relative lack of improvement in outcomes is that ventilation-based life support systems may have reached an efficacy threshold when used to support the extremely underdeveloped cardio-pulmonary system.^{6,7}

We have developed an experimental treatment platform for extremely preterm infants, *ex-vivo* uterine environment (EVE) therapy.⁸⁻¹¹ The central principle underlying the iterative development of this platform is to treat extremely preterm infants as fetuses, rather than as small babies, and in doing so avoid the use of pulmonary gas exchange. Although longer survival with a range of comparatively mature fetal models has been repeatedly demonstrated in sheep and goats over the past thirty years (broadly corresponding to 28-32 weeks gestation in human),^{9,12-15} the feasibility of using an artificial placenta-based system to successfully maintain extremely preterm

fetuses representative of the developmental stage of extremely preterm human
fetuses was only recently demonstrated for the first time.⁸

One significant question that has not been addressed over the 60 year history of
artificial placenta development is whether or not such a system could be used to
support compromised fetuses.⁸⁻¹⁵ This is an extremely important consideration given
that intrauterine infection and inflammation are common antecedents of extremely
early preterm labor (PTL), and likely impact the ability of a fetus to adapt to artificial
placenta-based treatments. It is reported that 40–70% of PTLs are associated with
chorioamnionitis, with prevalence correlated to fetal gestational age at preterm
delivery.^{16,17} For example, chorioamnionitis is observed in nearly 70% of deliveries at
24 weeks' gestation or earlier, but in only 16% of cases delivered at 34 weeks'
gestation.¹⁸

The presence of a fetal inflammatory response is strongly associated with not only
PTLs but also with a higher rate of fetal complications and death.^{19,20} In particular,
inflammation in response to microorganisms in the amniotic environment is
associated with life-threatening lung injuries represented by bronchopulmonary
dysplasia (BPD)^{21,22} and neonatal brain injuries represented by white matter injury
(WMI) or hemorrhage, resulting in the subsequent diagnosis of cerebral palsy
(CP).^{23,24} We suggest that for artificial placenta-based systems to be used clinically,
the technology must be capable of supporting extremely preterm fetuses
compromised by exposure to intrauterine inflammation.

We report the ability of our EVE therapy platform to support extremely preterm lambs (95 dGA; weighing approximately ~600g; approximately 24 weeks' GA in human equivalent) following exposure to intrauterine inflammation caused by intraamniotic (IA) exposure to *E.coli* lipopolysaccharide (LPS),^{25,26} for a period of 120 h. Although LPS elicits a sterile inflammatory response (as distinct from the use of viable microorganism) its use has the advantage of allowing for the generation of a well characterized and robust intrauterine inflammatory response, chorioamnionitis, and pathological changes in a number of fetal systems including the lung and brain.

As previously, a 120h maintenance period was chosen for this study based on clinical data showing the preponderance of death and injury identified in extremely preterm infants occurs acutely post-delivery. Additionally, in our experience, adverse outcomes in the EVE therapy model similarly occur within 2-3 days of establishment on the life support platform. The primary outcome measures were: **i)** maintenance of key physiological parameters; **ii)** absence of infection and inflammation; **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 and measurements 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). This study was comprised of two experimental groups:

- i) an IA LPS-exposed EVE Therapy Group (EVE Group) comprised of fetuses exposed to LPS for 2d prior to delivery and maintenance on EVE therapy for 5d (n=10); and
- ii) an IA LPS Control Group (LPS Control Group) comprised of fetuses remaining in utero for 7d post-LPS exposure (n=8).

Additional tissues from age matched, saline-exposed animals were obtained from our biobank^{8,26} to establish a third Saline Control Group (iii) to allow for an LPS-naïve comparison with LPS-exposed tissues.

Study Protocol

i) EVE Group: Ten merino-cross ewes (Term: ~150 d) with timed, singleton pregnancies were given single 10mg IA LPS injection under ultrasound guidance at 92-93dGA with successful amniotic fluid placement confirmed as described as previously^{25,26}. Fetuses were surgically delivered 48 hour later at 94-95 dGA and adapted to EVE Therapy as follows.

Surgical delivery

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.^{8-11,27} Ewes in both the EVE and the control groups were euthanized with an intravenous bolus of pentobarbitone (160 mg/kg). After a maternal laparotomy and hysterotomy, the fetuses were placed inside the sterilized artificial uterus, 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 8 Fr custom-made catheter (Nipro Corporation, Osaka, Japan) and secured approximately 1.5-2 cm external to the umbilical ring. The tip of the 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 Group 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 effusions or bleeding were determined by ultrasound (single operator), and by gross examination during necropsy after 120 h of EVE therapy.

Fetuses 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.²⁸ Midazolam was continuously administered for the first 6 h (0.15 mg/kg/hour).⁸ Hypoxia is characteristic for fetuses exposed to chorioamnionitis and a significant factor for severe brain injury. Oxygen supply to the membranous oxygenators was strictly adjusted to maintain fetal PaO_2 within a normal range between 15-30 mmHg.²⁹⁻³¹ 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).

Management of refractory hypotension

We defined refractory hypotension as hypotension resulting in a decrease of circuit flow below 150ml/kg/min (normal circuit flow is 150-250 mL/kg/min)³²⁻³⁴ which required glucocorticoid administration in addition to volume expansion (10-20ml/kg/hour) with fresh frozen plasma (FFP) to be resolved. To prevent critical refractory hypotension (which is frequently observed in extremely preterm infants at the border of viability), intravenous hydrocortisone (HC) (3 mg) was administered to all 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 described as previously.⁸ If refractory hypotension could not be controlled following primary administration of HC, a second dose of HC (3mg) and increased volume expansion (30ml/kg/ hour), and then finally intravenous dexamethasone (DEX; 0.15mg/dose, estimated 0.25mg/kg/dose; Hospira Australia, Melbourne, Australia) was provided. Hypotension and circuit flow were evaluated every 5-10 minutes after injection of HC or DEX. Iterative DEX was provided until hypotension and circuit flow were managed within normal range. FFP was provided for volume expansion according to hypotension for the first 4-6 h (10 - 30 mL/kg/hour) to all fetuses as described previously.⁸ Inotropic drug use was avoided so as to prevent contraction of the umbilical arteries.

Parental nutrition

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) was administered to provide 70-75 kcal/kg/day described as previously.⁸

Prevention of infection

Bacterial infection was determined based on a positive finding of bacteremia by microbial cultures. Meropenem (15 mg/kg/dose, Ranbaxy; Sydney, Australia) was administered intravenously to the fetuses every 6 h. Intravenous fluconazole (4 mg/kg/dose, Fluconazole-Clarix; AFT Pharmaceuticals Pty Ltd, Sydney, Australia) was administered to the fetuses every 24 h described as previously.⁸

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. 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 and warmed to physiological temperature 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-PGE₁ 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 m². Extracorporeal pumps were not used to maintain the circuit flow.

2) Amniotic Fluid

Synthetic AF was aseptically prepared as follows: pH: 7.34 ± 0.15 , Na⁺: 110 ± 4 mEq/L, Cl⁻: 111 ± 4 mEq/L, K⁺: 6.5 ± 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 $\pm$ 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 $38.7 \pm 0.3^\circ\text{C}$ (group mean $\pm$ SD). To minimize the risk of microbial colonization, a transparent, sterilized plastic bag was used to contain the AF bath. The AF bath was rinsed and replaced every 6 h after the start of the EVE therapy with 30 L of new synthetic AF.

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. Amniotic fluid was collected at surgical delivery. Fetal umbilical arterial blood samples were collected every 24 h following the induction of the EVE therapy.

Haematological analyses, including white blood cell counts (WBC), differential leukocyte counts, biochemical parameters; aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGTP), glutamate dehydrogenase (GLDH), blood urea nitrogen (BUN), total bilirubin, albumin, magnesium (Mg) and phosphorus (P) and BUN/creatinine ratio, endocrine parameters; Cortisol, adrenocorticotrophic hormone (ACTH), Insulin-like growth factor 1 (IGF-1) 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 to confirm normal fetal cardiac function at 48 h after LPS injection (before surgeries for EVE animals) and to assess fetal cardiac function 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 were held in a dorsal recumbency and the fetal position from the ventral aspect was confirmed. 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 described as previously.^{8,27}

Briefly, trans tricuspid and trans mitral inflow were measured using Doppler echocardiography to assess the peak early filling (E wave) and late diastolic filling (A wave) velocities in calculation of each E/A ratio. Cardiac time intervals such as isovolumetric contraction time (ICT), ejection time (ET), and isovolumetric relaxation time (IRT) were measured for the right and left ventricle. Myocardial performance index (MPI) was calculated using the formula defined as $MPI = ICT + IRT / ET$.

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, and the A/S ratio was calculated to obtain the preload index (PLI).

The inner diameter (D) of the aorta and the pulmonary artery was measured at an insonation angle perpendicular to the vessel wall, between the open semilunar valves.

The systolic time-velocity integral (TVI) and heart rate (HR) were calculated as an average of four to six cardiac cycles. Left and right ventricular output were calculated as $\pi \cdot (D/2)^2 \cdot TVI \cdot HR$. The cardiac output (CCO) was calculated as the sum of the two, and the corrected CCO was calculated by dividing this by the fetal weight.³⁵

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.

ii) LPS Control group

Eight merino-cross ewes with timed, singleton pregnancies were given IA LPS injection (10 mg) at 92-93 dGA as above. After 7 days of ultrasound assessments (as above) they were delivered and euthanized with an intravenous bolus of pentobarbitone (160 mg/kg) at 99 -100 dGA to allow comparative measurement of body weight (BW), crown rump length (CRL), organ weights (lung, brain), tissue collection (lung for PCR, brain for histopathological analysis), and fetal whole blood

and plasma collection at delivery (immediately prior to euthanasia) to perform blood corpuscle counts, including differential leukocyte count, biochemical and cytokine analyses.

iii) Saline Control Group (BioBank Samples)

Comparative analysis of AF and plasma MCP-1 concentrations between LPS-exposed and naïve fetal samples was performed using previously published, age matched samples obtained from saline-exposed catheterized fetuses drawn from our ovine bio bank²⁶. Saline Control Group blood gas data, WBC counts, differential leukocytes counts, plasma for cytokine, lung tissues for PCR and brain tissues for histopathology presented herein were similarly obtained as a reference set from our published ovine BioBank.⁸

Laboratory Analyses

Enzyme-Linked Immunosorbent Assays

MCP-1 protein concentrations were measured using fetal plasma and AF samples from EVE Group animals taken 48 after IA LPS injection.²⁶ Those values were compared with data from Saline Control and LPS Control group animals. At the end of the experimental period, fetal plasma concentrations of MCP-1 and Tumor necrosis factor alpha (TNF- α) was also compared between those of EVE animals and the Saline and LPS Control Group animals.

Commercial kits from Kingfisher Biotech (St. Paul, MN), with washing performed on a Biosan plate washer (3D-IW8, Inteliwasher, Biosan, Riga, Latvia) were used as

previously described.⁹ Standards (calibration curve $R^2 > 0.99$) 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 μ L 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).

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 described³⁶⁻³⁸. 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/ μ L.

Ovine-specific PCR primers and hydrolysis probes for interleukin (IL)-1 β , IL-6, 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 DNase-treated template fetal tissue RNA in a total volume of 20 μ L 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 guidelines³⁹. dCq values were used to perform statistical analyses for significant differences between intervention groups vs control group.

Preterm Brain Histology

Brains perfusion fixed with formalin were processed for gross anatomical investigation as described previously.⁸ 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 Larroche⁴⁰. 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.

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. In the comparison of more than two groups, between-group differences in parametric data were tested for significance with one-way ANOVA while Kruskal-Wallis tests were used for non-parametric data. Multiple post-hoc comparisons were performed with Turkey or Dunnett tests. All p values <0.05 were accepted as significant.

[Results]

Physiological variables

Eight of ten fetuses in the EVE Group and six of eight fetuses in the LPS Control Group completed a pre-determined 120 h experimental period (Table 1). Key physiological data are presented in Figure 1. One experimental fetus (Figure 1H) was excluded from general group comparison (Table 1) due to the presence of severe growth restriction (outlier; 494 g at 100 dGA). Another two fetuses were euthanized 24 h and 36 h after the initiation of EVE therapy due to acute circuit failure (catheter flow was obstructed by intense fetal movement) and were excluded from further analyses.

Fetal birth weight (BW) and lung weight were significantly lower and crown-rump length (CRL) was significantly shorter in EVE Group animals relative to LPS Control Group animals. There were no significant differences in gestational age, sex ratio, weight corrected CRL, weight corrected lung weight, brain weight and weight corrected brain weight at the conclusion of the 120 h study between the LPS Control and the EVE Group animals over the experimental period (Table 1).

For EVE Group animals, cord blood gas data had pO₂ values within the target range (15-30 mmHg). Other items; pH, pCO₂, BE, SO₂, O₂, CtO₂, Na⁺, K⁺, Ca²⁺, Cl⁻, hemoglobin, lactate level, glucose and activated clotting time also remained clinically acceptable and within an expected reference range (Table 2).

Statistically, AST, ALT, GGTP, GLDH, BUN levels and the BUN/creatinine ratio were modestly but significantly higher in EVE Group animals relative to those in the LPS Control group, while there was no difference in total bilirubin, albumin, Mg and P value. Creatinine level was significantly lower in EVE group than that in Control group at 120h, (Table 3). Similar findings were seen in our previous work with this model, and the biological relevance of these modest changes remains unclear.

Plasma cortisol concentrations in EVE Group animals were significantly increased over the 120 h period of EVE therapy compared with values taken at the start of experiments (0 h). Cortisol concentrations at the end of experiment in EVE Group animals were significantly higher than those in the LPS Control Group animals (Figure 2 A). Plasma concentrations of ACTH in EVE Group animals were significantly decreased over the 120 h experimental period, relative to values measured at the start of experiments (0 h). ACTH concentrations taken at the end of experiments (120 h) in EVE Therapy Group animals were significantly lower than those in the LPS Control Group animals (Figure 2 B). In EVE Group animals, plasma concentrations of IGF-1 were significantly decreased at 48, 72, 96, 120 h compared to measurements taken at 0 h. IGF-1 concentrations at the end of experiment in EVE Group animals were significantly lower than in the LPS Control Group animals (Figure 2 C).

Ultrasound analysis for viability 48 h after LPS injection confirmed fetal cardiac function in all LPS Control and EVE Group animals. For ultrasound assessments performed before euthanasia, there was no significant difference in Tricuspid and Mitral valve E/A ratio, Right and Left MPI, PLI and corrected CCO between the LPS Control Group and EVE Group animals. The direction of flow through the *ductus arteriosus* was from right to left in each of eight EVE animals (Table 4).

Individual case information (Figure 1 A-H) is as follows, with data summarized in Table 5.

EVE Group Animals 1 and 2 (Case A, B): 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 WMI) were not detected histopathologically. No bacteria were identified from blood and AF cultures.

EVE Group Animal 3 (Case C): Blood pressure and total circuit flow sharply decreased from 1 h after induction of EVE therapy and was controlled by three doses of dexamethasone (total = 0.45 mg) in addition to predetermined treatment (volume load and primary administration of HC). All measured physical parameters were subsequently maintained within respective reference ranges for the first 60 h. Normal intermittent active fetal swallowing movements, breathing movements, gross fetal

body movements and flexure and extension of limbs were observed in first 60 h. Circuit flow was interrupted for approximately five minutes with an unexpected occlusion of catheter flow at 60 h after EVE therapy. Circuit flow was restored with change of catheter position and angle. 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. There was no evidence of pleural effusion and bleeding, while there was slight edema and ascites. Review of H&E-stained coronal sections (mammillary bodies 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.

EVE Group Animal 4 (Case D): Blood pressure and total circuit flow sharply decreased from 30 minutes after induction of EVE therapy and was controlled by three doses of dexamethasone administration (total = 0.45 mg) in addition to predetermined treatment. All measured physical variables subsequently 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. 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.

EVE Group Animal 5 (Case E): Blood pressure and total circuit flow sharply decreased from 1.5 h after induction of EVE therapy and was controlled by two doses of dexamethasone administration (total = 0.3 mg) in addition to predetermined treatment. All measured physical variables subsequently 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. There was no edema, ascites, pleural effusion and bleeding. There were no histopathological brain injuries. No bacteria were identified from blood and AF culture bottles.

EVE Group Animal 6 (Case F): Blood pressure and total circuit flow sharply decreased from 0.5 h after induction of EVE therapy and was controlled by two doses of dexamethasone administration (total = 0.3 mg) in addition to predetermined treatment. All measured physical parameters were subsequently maintained within the respective reference ranges in first 108 h. Normal intermittent active fetal swallowing movements, breathing movements, gross fetal body movements and flexure and extension of limbs were observed in first 108 h. After this time circuit flow started to fluctuate in response to fetal active movements and posture changes. 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. There was no ascites, pleural effusion and bleeding, while there was slight edema. There were no histopathological brain injuries. No bacteria were identified from blood and AF culture bottles.

EVE Group Animal 7 (Case G): Blood pressure and total circuit flow sharply decreased from 0.5 h after induction of EVE therapy and was controlled by two doses of dexamethasone administration (total = 0.3 mg) in addition to predetermined treatment. All measured physical variables subsequently 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 though *Pseudomonas stutzeri* was identified in synthetic AF culture bottles taken both from artificial uterus and the synthetic amniotic fluid reservoir (prior to administration to the experimental system) at the conclusion of the experiment.

EVE Group Animal 8 (Case H): This fetus had growth restriction, with a fetal BW (494g), height (24cm), weight corrected brain weight (45.3g/kg) at conclusion of the study. Blood pressure and total circuit flow sharply decreased from 0.5 h after induction of EVE therapy and was controlled by two doses of dexamethasone administration (total = 0.6 mg) in addition to predetermined treatment. All measured physical variables subsequently 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. 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, although *Pseudomonas stutzeri* was identified in synthetic AF culture bottles taken both from artificial uterus and the synthetic amniotic fluid reservoir (prior to administration to the experimental system) at the conclusion of the experiment.

EVE Therapy Group Infection and Inflammation

The plasma concentration of MCP-1 in LPS Control and EVE Group animals was significantly higher than that of Saline Control Group animals, while there was no difference in between EVE Therapy and LPS Control Group animals (Figure 3 A). Similarly, the AF concentration of MCP-1 in LPS Control and EVE Group animals was significantly higher than that of Saline Control Group animals (Figure 3 B).

Evaluation of infection and inflammatory response at the end of experimental period (120h after EVE or 168 h after LPS injection for the Control animals)

No bacteremia was demonstrated from the blood cultures in any of the eight EVE Therapy Group animals, though *Pseudomonas stutzeri* was grown from the AF cultures in two EVE Group animals (Case G,H) at the end of the experimental period (120 h). There was no significant difference in plasma concentrations of MCP-1 between Saline Negative Control, LPS Control and EVE Group animals (one-way ANOVA; $p=0.372$) and plasma TNF- α was not detected in any of the groups (data not shown).

The number of circulating WBCs in LPS Control and EVE Group animals was significantly higher than that of Saline Negative Control Group animals, while there

was no difference between LPS Control and EVE Group animals. Similarly, the number of neutrophils in the LPS Control Group and EVE Group animals was significantly higher than that of Saline Negative Control Group animals; there was no difference between LPS Control Group and EVE Group animals. There was no difference in the number of lymphocytes between any of the groups. The number of monocytes was higher in LPS Control Group animals compared to both the Saline Negative Control and EVE Group animals (Figure 4 A).

For lung qPCR, no significant differences in the relative mRNA expression of IL-1 β , 6, TNF- α were identified between Saline Negative Control, LPS Control and EVE Group animals. The relative mRNA expression of MCP-1 in LPS Control Group animals was significantly higher than Saline Negative Control and EVE Group animals. (Figure 4 B).

Brain histopathology

Three LPS Control Group animals and three EVE Group animals had WMI in H&E-stained coronal sections (anterior basal ganglia level or mammillary bodies level), while there was no WMI in any of nine Saline Negative Control Group animals. There was no evidence of haemorrhage or WMI in any of the remaining LPS Control Group, EVE Group or Saline Negative Control Group animals in gross anatomical and H&E stained histopathological analyses of coronal brain sections. (Figure 5).

[Comment]

The primary finding of this study is that EVE Therapy allowed a 120 h period of survival in a group of eight extremely preterm lambs compromised by a 48h antenatal exposure to intrauterine inflammation (Figure 1, 3, 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 (400-700 g), compromised by exposure to intrauterine inflammation - a common antecedent of extremely preterm delivery. Fetal growth restriction was seen in EVE Therapy Group animals relative to LPS Positive Control (in utero) Group animals, although EVE Therapy Group animals were otherwise stable from a haemodynamic viewpoint after acute intensive management (Figure 1, Table 1-3,5) and did not present evidence of altered cardiac parameters by ultrasound (Table 4). This is a particularly important finding as, ideally, fetal growth should be maintained as close to normal as possible on an artificial placenta platform. Given the differences in somatic growth and hormone concentrations identified in the three study groups, it appears that the observed growth restriction derives from an as-yet unidentified deficiency in our EVE-therapy system, rather than LPS exposure itself.

All animals were free of bacteraemia and no additional systemic inflammatory changes were detected at conclusion of treatment in the EVE Group animals, relative to LPS Control Group animals (Figures 3, 4 Table 5, 6). Although a treatment time of

120 h may be limited in terms of clinical use, data from this study shows the potential of EVE therapy as a clinical platform for extremely preterm infants exposed to intrauterine inflammation.

Maintenance of key physiological parameters

Key physiological parameters and blood lactate levels generally remained within their reference ranges, or rapidly returned to reference range after EVE therapy was started and/or refractory hypotension was successfully treated (Figure 1, table 2). Total lung flow volume was maintained within normal range (150-300 mL/kg/min) which was similar to that shown by our previous study using extremely preterm lamb model.⁸ Although fetal hypoxia ($pO_2 < 20$ Torr) is likely to be induced by LPS and reported to become a factor of brain injury^{41,42} pO_2 and CtO_2 was maintained within a reference range at least after the induction of EVE therapy (Table 2). However, refractory hypotension and following transient elevation of lactate level might reflect fetal ischemic changes (Figure 1).

Ultrasound data

The main aim of these ultrasound measurements was the determination of cardiac dysfunction in EVE therapy animals and reference range was described in previous report using extra preterm lamb model.⁸ Differences in any items between LPS Control and EVE Group animals were not demonstrated, and value of all items in both group animals were within reference range. Fetal circulation (R→L direction through ductus arteriosus) was maintained with EVE therapy over time 120 h experimental period.

Infection and inflammation

All animals were free of bacteraemia throughout the experiment. In terms of inflammation at 48 after IA LPS injection, MCP-1 has been reported to be significantly increased both in plasma and AF for extremely preterm fetus after IA LPS injection,^{25,26} was evaluated as an indicator of inflammation. EVE Therapy Group animals had a significant increase of MCP-1 which was equivalent to that of LPS Positive Control Group animals (Figure 3 A, B). Thus, intrauterine inflammation was induced by IA LPS injection, and EVE surgery and platform maintenance could be conducted using extremely preterm lambs compromised by intrauterine inflammation. WBC and neutrophil levels increased both in LPS Control Group animals and EVE Group animals, and were equivalent at the end of the 120h experimental period (Figure 4 A). Reportedly, in preterm sheep, peripheral WBC and neutrophil levels increase over 4-7 days after exposure to IA LPS. WBC counts then return to control levels by 15 d, while neutrophils counts remain increased at 14 d after LPS exposure.⁴³⁻⁴⁵ Thus, this increase of WBC and neutrophils seemed not to be induced by additional infection but by the IA LPS injection.

MCP-1 transcript expression in lung tissue of LPS Control Group animals was significantly higher than that of Saline Negative Control Group animals and EVE Group animals at the conclusion of experiment, while all other measured transcripts had returned to baseline (Figure 4 B). In preterm fetal sheep, IA LPS injection induces large increases in pro-inflammatory cytokine responses in the fetal lung.^{25,26} Those inflammatory responses (IL-1 B, IL-6, TNF- α) are reported to return to baseline by 7-15 days after IA injection ⁴⁵, and our data suggest that adaptation of compromised

extremely preterm fetuses to EVE therapy does not exacerbate existing inflammation or gross brain injury.

Success rate

The survival rate with EVE therapy was 80% while that with the Control animals was 75% (Table 6). Two EVE animals died due to catheter-based circuit compromise. Two Control animals also died due to unidentified reasons. Reportedly, intravenous LPS injection causes fetal systemic inflammation, finally leading to fetal death.⁴⁶ However, fetal death due to IA LPS injection has not been reported in extremely early GA experiments, although this may be due to more abbreviated study periods being reported^{25,26,47}. In terms of the present study, no fetal death was detected by ultrasound in either the LPS Positive Control or EVE Therapy Group animals during the first 48 h after IA LPS injection.

Limitations

The primary limitation of this study is the small sample number and short trial period described. As previously, a 5 day maintenance period was selected based on several considerations. Firstly, to balance the substantial amount of infrastructure and constant monitoring, which limits the sample number achievable.⁸ Secondly, as this was the first attempt to adapt extremely early gestational fetuses compromised by intrauterine inflammation to an artificial placenta system, we elected to limit to

duration of the experiment to 5 days, allowing us to establish the acute efficacy of our system as described previously.⁸

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 comprehensive assessments of organ development. It is also, given the nature of the experimental design, not possible for us to assess the haemodynamic impact of IA LPS on LPS Control Group animals without incorporating additional surgical interventions. We elected not to pursue this approach in the present study as we wished to focus on a comparison of fetal development in the absence of potential confounding from fetal surgery necessary to introduce catheters and monitoring probes; future studies may require the addition of an instrumented LPS Control Group in addition to a non-instrumented group.

The use of glucocorticoids appears essential for current EVE therapy in maintenance of extremely preterm lambs at the border of viability, while it is also well known that the use of glucocorticoids to premature fetuses affects maturation of vital organ such as brain and lung, inflammatory markers and death.⁴⁸⁻⁵¹ It is also worth noting that glucocorticoids are also effective anti-inflammatory agents – which should be taken into account when assessing the inflammatory data presented in this study. This is also of importance as the LPS Control Group animals were not exposed to steroids, or antibiotics, both of which have the potential to modulate inflammatory and injury responses. Given the potential for glucocorticoid-induced changes to fetal development, extensive assessments of this optimal dosing and the impact of

glucocorticoids on inflammation and growth will be a key element of future studies; such analyses will be facilitated by long-term studies using our refined EVE Therapy system, and will be essential to demonstrate the clinical utility of this concept for the management and support of extremely preterm infants.

An additional limitation of importance is the inflammatory stimuli employed. The present study used LPS, which is a sterile modulator of toll-like receptor 4 signaling. It has the advantage of delivering a standardized, albeit self-limited pro-inflammatory response. However, it does not faithfully replicate the polyvalent stimulation derived from an active microbial or fungal infection of the uterus, nor does it facilitate fetal sepsis, which is similarly associated with extremely preterm delivery.^{52,53} Delivery timing was set at 48 h after IA LPS injection on the basis of previous studies.^{25,26,44,45} Although this setting would enable us to evaluate if induction of EVE therapy using extremely preterm lambs exposed to intrauterine inflammation was feasible, it might not be appropriate to evaluate about the resolution of fetal inflammations as many inflammatory responses induced by IA LPS return to the standard level in 7 d.^{44,45}

Challenges yet to be overcome for clinical application

Refractory hypotension

In the present study, HC was administered to manage refractory hypotension after induction of EVE therapy.⁸ However, hypotension (which could be managed comparatively easily in previous studies using LPS-naïve fetuses) could not be easily controlled even with the use of additional volume expansion and administration of HC in the majority of EVE Group animals exposed to intrauterine inflammation (EVE 1-8).

1 Profound refractory hypotension in a number of these animals occurred within first 3 h
2 after induction of EVE therapy. Although total lung flow and blood pressure were
3 initially maintained within a normal range after adaptation to EVE therapy, both
4 metrics started to degrade shortly thereafter.

5
6 Hypotension is frequently identified in preterm infants, and its incidence is inversely
7 related to GA at delivery likely due to a host of factors such as immature myocardium,
8 immature autonomic nervous system and relative adrenal insufficiency.⁵⁴⁻⁶¹ About
9 25% of ELBW infants with hypotension do not respond to either volume or inotropic
10 drug, and are treated by HC, independent of serum cortisol concentrations, to
11 normalize blood pressure.⁶²⁻⁶⁴ In our study HC treatment and volume expansion was
12 insufficient for a number of EVE Therapy Group animals exposed to intrauterine
13 inflammation to recover from refractory hypotension. Although the mechanism for this
14 is unclear, it may be because chorioamnionitis also releases inflammatory mediators
15 which increase vascular permeability and vasodilation, and in doing so predispose
16 the fetus to refractory hypovolemia or hypotension.⁶⁴ Administration of the strong
17 glucocorticoid dexamethasone might improve the clinical picture by compensating for
18 relative steroid insufficiency as well as contributing to reduced inflammation via its
19 anti-inflammatory activity. Given the time-scale involved, it is also possible that
20 several hours are required for the benefit of glucocorticoid signaling changes to
21 translate into improvements in physiological parameters, and that additional steroid
22 administration (including DEX) is unnecessarily excessive.

Although the optimal dose and timing remains unclear, HC and DEX co-administration assisted us in achieving 120 h survival time for eight in ten EVE Group animals exposed to intrauterine inflammation. However, EVE Group animals still had transient increases of lactate level after recovery from hypotension. HC and DEX therapy resulted in high concentrations of fetal cortisol and may have suppressed ACTH secretion (Figure 2 A, B). Thus, optimization of appropriate timing and dose, and evaluation of long-term effects of glucocorticoid treatment will become key elements of future work to minimize adverse effect for the growth or neurodevelopment

Growth Restriction

EVE Group animals had growth restriction compared to LPS Control animals. LPS Control Group animals had ostensibly normal growth⁸ despite being subjected to the same IA LPS exposure as growth-restricted EVE Group animals. These data suggest that EVE Group animals exposed to intrauterine inflammation are susceptible to extra uterine growth restriction (EUGR), the most frequent morbidity among premature babies especially < 1500g under current neonatal clinical settings.^{65,66}

The primary cause of EUGR is widely considered to be nutrient insufficiency.^{67,68} Although EVE Group animals were given the same nutrient supplementation described as previously,⁸ that level of support may not meet the requirements of extremely preterm lambs exposed to intrauterine inflammation. The metabolic response to and nutritional requirements of sepsis in the neonate are not well-defined; septic neonates are reported to require higher energy delivery during the acute phase of their illness. In adults, sepsis alters protein requirements more

1 acutely by its effect on cytokine-mediated muscle catabolism. A similar requirement
2 may be seen in extremely preterm fetuses. No study has assessed whether provision
3 of extra protein will reduce this catabolic response for neonates, but at this point it
4 seems prudent to provide at least 2.5 g/kg per day of protein to the septic infant.^{69,70}
5 Thus, optimization of appropriate nutrition for extremely preterm infants with
6 intrauterine inflammation may be an important key for future treatment.

7
8 HC and DEX use also might be a cause of growth restriction. The use of
9 corticosteroids such as HC and DEX has been established to prevent or reduce
10 chronic lung injury for premature babies. However, neonatal DEX has also been
11 associated with impairment of somatic growth, both during treatment ('early growth
12 restriction')^{71,72} and at follow-up of formerly DEX-treated newborns at ages ranging
13 between 2 and 17 years⁷³⁻⁷⁶, while no significant effect of postnatal HC therapy on
14 somatic growth at ages ranging 2–17 has been found.⁷⁷⁻⁸⁰ Although It is presently
15 unclear which impact the combined effects of preterm birth and corticosteroid-related
16 early growth retardation may have on subsequent growth; whether
17 glucocorticoid-treated babies sometime demonstrate catch-up growth is presumed to
18 underlie long-term health risks.⁸¹ Appropriate use of glucocorticoids will be key for
19 future studies in this area (Figure 2 A, B).

20
21 IGF-1 values in EVE Group animals were decreased over the experiment and were
22 significantly lower than that of the LPS Control Group animals at the experiment
23 conclusion (Figure 2 C). Whereas IGF-2 is the primary growth factor underpinning
24 embryonic growth, the dominant fetal growth regulator is IGF-1, and is produced

mainly by the fetal liver in addition to amniotic fluid and other organs.⁸²⁻⁸⁴ Umbilical cord IGF-I levels correlate closely with birth weight and there has been a report of one human patient with an IGF-1 gene deletion who presented with severe IUGR.^{85,86} Similarly, direct fetal infusion of IGF-1 promotes fetal substrate uptake and inhibits fetal catabolism and influences.⁸⁷ In contrast to the postnatal situation, the dominant driver of circulating fetal IGF-1 regulator is not only growth hormone (GH),⁸⁸⁻⁹⁰ but also fetal insulin, which in turn is predominantly under regulation by fetal glucose availability.^{91,92} Thus, in this study, inappropriate nutrition (inappropriate glucose or insulin level), excessive corticosteroid dose (especially DEX) which might interfere with GH pulsation and decrease total GH secretion and IGF-1,^{93,94} the absence of amniotic fluid derived IGF-1 may combine to result in an overall deficiency of IGF-1 levels and become a factor in growth restriction. Therefore, monitoring IGF-1 and IGF binding proteins⁹⁵ as an indicator of fetal growth in management of nutrition and GC use or replenishing IGF-1 may improve outcomes in this experimental system.

Brain injury

Gross anatomical observations and assessment of H&E stained brain sections identified the presence of pathological lesions in three of the LPS Control Group animals and three of eight EVE Group animals (EVE 3, 4, 8).

There was no evidence of haemorrhage in any of the six LPS Control and eight EVE Group animals, or of WMI in the remaining three control and six EVE animals in gross anatomical and H&E stained histopathological analyses of coronal brain sections. (Table 5, Figure 5).

WMI has been associated with prematurity especially below 32 weeks GA, typically localized to the periventricular white matter area in a diffuse or focal pattern.^{96,97} Additionally, inflammation during fetal and neonatal development alone, has been linked to brain injury.⁹⁸⁻¹⁰⁰ Similarly, extremely preterm fetal sheep (91- 93d GA) exposed to LPS reported WMI.^{101,102} Although the cause of this injury in this study is unclear, it might derive from hypoxic-ischemic change or pro-inflammatory cytokines mediating direct effects.^{8,30,103-106}

[Conclusions]

Extremely preterm sheep fetuses compromised by a 48h antenatal exposure to intrauterine inflammation were maintained for a period of 120 h period using an artificial placenta-based life support platform. These studies have identified four key issues for the future development of this technology for clinical application: **i)** refractory hypotension; **ii)** growth restriction; **iii)** brain injury; and **iv)** stabilizing circuit performance to protect against white matter injury.

As such challenges are largely absent from studies based on healthy sheep fetuses, additional experiments of this nature are essential for further development of this technology. Although still preliminary, these novel findings using extremely preterm lambs compromised by exposure to intrauterine inflammation do demonstrate that, with significant additional development, there is potential clinical utility for artificial placenta technology to support extremely preterm infants.

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	LPS Control Group	EVE Group	Statistical test	p value
Planned number	8	10		
Survival number	6	8		
Number for statistics	6	7		
Gestational age at the LPS injection (d)	92.8 ± 0.8	92.1 ± 0.7		
Gestational age at the induction of EVE therapy (d)	-	94.1 ± 0.7		
Gestational age at the conclusion (d)	99.8 ± 0.8	99.1 ± 0.7	t-test	0.122
Sex (male/female)	0.5	3/4	Chi-square test	0.383
Body weight (g)	1051 [973 - 1090]	869 [816-883] *	Mann-Whitney U test	0.035
Crown rump length (cm)	32.1 ± 1.0	30.3 ± 1.2*	t-test	0.013 [-3.14 - -0.46]
Weight corrected crown rump length (cm/kg)	31.1 ± 1.8	34.5 ± 4.5	t-test	0.118
Lung weight (g)	38.7 ± 1.2	34.8 ± 2.5*	t-test	0.004 [-6.33 - -1.5]
Weight corrected lung weight (g/kg)	37.6 ± 2.7	39.6 ± 5.7	t-test	0.444
Brain weight (g)	26.5 ± 1.6	25.1 ± 1.0	t-test	0.093
Weight corrected brain weight (g)	25.1 [23.3 - 27.0]	28.7 [22.6 - 30.0]	Mann-Whitney U test	0.101

Table 1. Comparison of fetal data at necropsy. Respective values are expressed as the group mean ± SD and significant p values with [95% confidence interval] were indicated for t-test. Respective values are expressed as mean [IQR] for Mann-Whitney U test. Significant difference vs. value for the Control group is indicated: *, p<0.05

	Reference	EVE
pH	7.38 ± 0.03	7.39 ± 0.05
Base excess (mmol/L)	-0.2 ± 1.8	0.8 ± 3.6
pCO ₂ (Torr)	42.4 ± 2.9	43.6 ± 3.8
pO ₂ (Torr)	25.1 ± 2.1	27.9 ± 3.8
SO ₂ (%)	65.5 ± 5.9	64.4 ± 7.9
CtO ₂ (mmol/L)	8.9 ± 1.2	9.2 ± 1.3
Hemoglobin (g/L)	93 ± 9	105 ± 5
Glucose level (mmol/L)	1.6 ± 0.2	3.2 ± 1.3
Lactate level (mmol/L)	1.7 ± 0.4	1.6 ± 0.9
Na ⁺ (mmol/L)	134 ± 3.1	143 ± 5.7
K ⁺ (mmol/L)	4.0 ± 0.3	3.5 ± 0.5
Ca ²⁺ (mmol/L)	1.4 ± 0.1	1.3 ± 0.1
Cl ⁻ (mmol/L)	103 ± 1.7	112 ± 6.0
Activated clotting time (sec)	-	203 ± 24

Table 2. Blood gas data throughout experiments in the EVE group.

Eight EVE Group 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 $\pm$ SD. SO₂, O₂ saturation; CtO₂, O₂ content = hemoglobin (g/dl) $\times$ 1.34 $\times$ SpO₂ (%) / 100 + pO₂ $\times$ 0.003; Na⁺, sodium ion; K⁺, potassium ion; Ca²⁺, calcium ion; Cl⁻, chloride ion. The reference data were obtained from thirteen age-matched (97 $\pm$ 2 dGA), null-treatment fetuses, which were previously collected for our ovine databank.

	LPS Control Group	EVE Group	Statistical test	p value
AST (U/L)	24 [22 - 28]	56 [40 - 138] *	Mann- Whitney U test	0.003
ALT (U/L)	3.0 [2.2 - 3.0]	9.5 [6.8 - 5.0] *	Mann- Whitney U test	0.008
GGTP (U/L)	22 ± 9	48 ± 16 *	t-test	0.012 [7 - 45]
GLDH (U/L)	10 [5 - 17]	17[40 - 138] *	Mann- Whitney U test	0.012
Total bilirubin (mg/dl)	1.9 ± 0.5	2.4 ± 1.9	t-test	0.105
Albumin (g/dl)	1.9 ± 0.1	2.0 ± 0.2	t-test	0.247
BUN (mg/dL)	13.5 ± 3.2	20.3 ± 6.5 *	t-test	0.03[0.8 - 12.8]
Creatinine (mg/d/L)	0.87 ± 0.14	0.53 ± 0.17 *	t-test	0.001[-0.53 - -0.16]
BUN/Cre ratio	15.7 ± 4.6	38.0 ± 4.1 *	t-test	0.001[17.9 - 29.9]
Mg (mmol/L)	1.2± 0.2	1.1 ± 0.1	t-test	0.397
P (mmol/L)	2.2± 0.3	2.0 ± 0.2	t-test	0.07

Table 3. Comparison of fetal chemical data at 120h. Six LPS Control Group animals and eight EVE Group animals were analyzed.

Respective parametric values are expressed as the group mean ± SD and significant p values with [95% confidence interval] were indicated for t-test. Respective nonparametric values are expressed as mean [IQR] for Mann-Whitney U test. Significant difference vs. value for the Control group is indicated: *, p<0.05. AST, aspartate aminotransferase; ALT, alanine aminotransferase, GGTP, gamma-glutamyl transpeptidase; GLDH, glutamate dehydrogenase; BUN, blood urea nitrogen; Mg, magnesium; P, phosphates.

	LPS Control Group	EVE Group	p value
Tricuspid valve E/A ratio	0.72 ± 0.08	0.68 ± 0.08	0.414
Mitral valve E/A ratio	0.68 ± 0.11	0.66 ± 0.08	0.774
Right myocardial performance index	0.43 ± 0.04	0.43 ± 0.08	0.417
Left myocardial performance index	0.41 ± 0.03	0.42 ± 0.03	0.99
Preload index	0.35 ± 0.12	0.38 ± 0.14	0.67
Corrected cardiac output (ml/min/kg)	530 ± 60	511 ± 122	0.74
Direction of ductus arteriosus flow	Right → Left	Right → Left	

Table 4. Comparison of fetal cardiac ultrasound data at study conclusion. Six LPS Control Group animals and Eight EVE Group animals were analyzed. Respective 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.

EVE animals	EVE1 (A)	EVE2 (B)	EVE3 (C)	EVE4 (D)	EVE5 (E)	EVE6 (F)	EVE7 (G)	EVE8 (H)
swallowing movement	+	+	(24–62H) +	+	+	(24–108H) +	+	+
breathing movement	+	+	(24–62H) +	+	+	(24–108H) +	+	+
gross body movements	+	+	(24–62H) +	+	+	(24–108H) +	+	+
flexure and extension of limbs	+	+	(24–62H) +	+	+	(24–108H) +	+	+
edema	–	–	+	–	–	+	–	–
ascites	–	–	+	–	–	–	–	–
pleural effusions	–	–	–	–	–	–	–	–
Total dose of dexamethasone administration in control of refractory hypotension (mg)	–	–	0.45	0.45	0.3	0.3	0.3	0.6
brain bleeding	–	–	–	–	–	–	–	–
White Matter Injury	–	–	+	+	–	–	–	+
blood culture	–	–	–	–	–	–	–	–
Culture from amniotic fluid in artificial womb	–	–	–	–	–	–	<i>Pseudomonas stutzeri</i>	<i>Pseudomonas stutzeri</i>
Culture from amniotic fluid in Tub	–	–	–	–	–	–	<i>Pseudomonas stutzeri</i>	<i>Pseudomonas stutzeri</i>
specified issue	–	–	circuit trouble due to a kink of the tip of the catheters	–	–	circuit trouble due to a kink of the tip of the catheters		Severe fetal growth restriction (weight=494g, height=24cm, weight corrected brainweight = 45.3 g/kg at conclusion)

Table 5. Case summary of EVE Group animals. +, present; –, absent. 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 culture bottles were collected from fetal umbilical artery, synthetic AF in artificial womb and sterilized tab at the end of the experimental period.

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	LPS Control Group	EVE Group
Total Number	8	10
Survival number	6	8
Survival rate (%)	75	80
Completion number without bacteremia among survived animal	-	8
Completion rate without bacteremia among survived animal (%)	-	100
Completion number without brain injury among survived animal	3	5
Completion rate without brain injury among survived animal (%)	50.0	62.5

Table 6. Success rate over time in the EVE Group. Bacteremia was determined by microbial culture. Brain injury was determined by the presence of IVH, WMI identified in gross anatomical observation and histological assessment of the brain sections.

[Figure legends]

Figure 1. 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. Black arrows show the timing of refractory hypotension.

Figure 2. Changes in the concentrations of cortisol, ACTH and IGF-1 over time in the EVE Group and comparisons between EVE and Control groups at the end.

ACTH; adrenocorticotrophic hormone, IGF; Insulin-like growth factor 1

Six LPS Positive Control animals (CT) and eight EVE animals (EVE) were analyzed plasma concentrations of cortisol (Panel A), ACTH (Panel B) and IGF-1 (Panel C).

The horizontal axis represents the time after the induction of the EVE therapy (h). All values are presented as bar charts with the group mean and with whiskers representing SD. Gray bar indicates EVE animals. Black bar indicates Control animals. Differences of values over time in EVE animals were tested for significance using repeated one-way ANOVA. Multiple post-hoc comparisons were performed using Dunnett test compared with the value at 0 h; * indicates p value <0.05 accepted as significant.

Differences of values at the end of experimental period between EVE animals and Control animals were tested using t-test; † indicates p value <0.05 accepted as significant.

Figures 3. Comparison of concentration values of monocyte chemoattractant protein 1 (MCP-1) in fetal plasma and AF at 48 h after LPS injection

Nine Saline Negative Control Group animals (saline treatment was conducted at 93dGA), nine LPS Control Group (LPS treatment was conducted at 93dGA) and eight EVE Group animals (LPS treatment was conducted at 93dGA) were analyzed.

Panel A shows the comparison of fetal plasma concentration and Panel B shows the comparison of AF concentration of MCP-1. All values are presented as bar charts with the group mean and with whiskers representing SD. The white bars indicate negative control group. The black bars indicate positive control group. The gray bars indicate the EVE group. Differences of values between the groups were tested for significance using one-way ANOVA. Multiple post-hoc comparisons were performed using Turkey † indicates p value <0.05 accepted as significant.

Figure 4. Differential Cell Counts (blood) and Cytokine mRNA Expression (lung) at the end of the experiment.

Nine Saline Negative Control Group (saline treatment; Data taken from our ovine bank age-matched at 99.9 d GA $\pm$ 0.7 d), six LPS Control Group (LPS treatment animals) and eight EVE Group animals were analyzed.

In panel A; WBC, white blood corpuscle. All values are presented as bar charts with the group mean and with whiskers representing SD. The white bars indicate the

Saline Negative Control group. The black bars indicate the LPS Control Group. The gray bars indicate the EVE Group. Differences of values between the groups were tested for significance using one-way ANOVA. Multiple post-hoc comparisons were performed using Turkey † indicates p value <0.05 accepted as significant.

In panel B; TFNA: tumor necrosis factor α , MCP1; monocyte chemoattractant protein 1. Relative fold changes in cytokine (Interleukins-1 β , 6, tumor necrosis factor α and monocyte chemoattractant protein 1) mRNA expression in lung tissue samples. All values are presented as box plots with the group median and with whiskers representing maximum and minimum values. White box indicates Saline Negative Control Group and black box indicates LPS Control Group animals. Gray box indicates EVE Group animals. Respective differences of values were tested for significance using Kruskal- Wallis test. Multiple post-hoc comparisons were performed using Turkey † indicates p value <0.05 accepted as significant.

Figure 5. Representative images of brain histology.

Six LPS Control Group animals and eight EVE Group animals (EVE 1-8) were analyzed with H&E stain. 6 fields from four 5mm serial sections were assessed for each animal. Three Control animals and three EVE animals (EVE 3, 4, 8) had white matter injury (WMI). Representative image of WMI from one of injured LPS Positive Control animals (Panel A, D), EVE 4 in EVE animals (Panel B, E) and representative image of non WMI from Saline Negative Control Group animals (Panel C, F) identified in investigation with H&E staining showing necrosis and cellular infiltration. Images are inspected at 100 x total magnification (Panel A-C) and 200 x (Panel C-F)); scale bar represents 100 μ m.