OBSTETRICS

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

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BACKGROUND: Ex vivo uterine environment 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 ex vivo uterine environment therapy 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.

OBJECTIVE: The objective of the study was to use our ex vivo uterine environment therapy platform to support the healthy maintenance of 600−700 g/95 days gestational age (equivalent to 24 weeks of human gestation) sheep fetuses. Primary outcome measures were as follows: (1) maintenance of key physiological variables; (2) absence of infection; (3) absence of brain injury; and (4) growth and cardiovascular function patterns matching that of noninstrumented, age-matched in utero controls.

STUDY DESIGN: Singleton fetuses from 8 ewes underwent surgical delivery at 95 days' gestation (term, 150 days). Fetuses were adapted to ex vivo uterine environment therapy and maintained for 120 hours 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 euthanized at 100 days' gestation to allow comparative postmortem analyses. Data were tested for mean differences with an analysis of variance.

RESULTS: Seven of 8 ex vivo uterine environment group fetuses (87.5%) completed 120 hours of therapy with key parameters maintained in a normal physiological range. There were no significant intergroup differences ($P > .05$) in final weight, crown-rump length, and body weight-normalized lung and brain weights at euthanasia compared with controls. There were no biologically significant differences in hematological parameters (total or differential leucocyte counts and plasma concentration of tumor necrosis factor-α and monocyte chemoattractant protein 1) ($P > .05$). Daily blood cultures were negative for aerobic and anaerobic growth in all ex vivo uterine environment animals. There was no difference in airspace consolidation between control and ex vivo uterine environment 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-1, interleukin-6, interleukin-8, tumor necrosis factor-α, and monocyte chemoattractant protein 1 mRNA expression in lung tissues compared with the control group. No cases of intraventricular hemorrhage were observed, and white matter injury was identified in only 1 ex vivo uterine environment fetus.

CONCLUSION: 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 hours. With additional refinement, the data generated by this study may inform a treatment option to improve outcomes for extremely preterm infants.

Key words: artificial placenta, extremely preterm infants, ex vivo uterine environment therapy, fetal brain injury, fetal inflammatory responses

More than 1 million babies die as a result of prematurity each year worldwide.[1] Overall, outcomes for preterm infants have markedly improved over the past 5 decades as a result of improvements in neonatal and obstetric care including antenatal steroid therapy, and the postnatal 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 1 or more severe, life-compromising disabilities.[2] Outcomes of extremely premature (<28 weeks' gestation) and extremely low birthweight infants (ELBW; <1000 g) have changed little for at least these 2 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 cardiopulmonary 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 (ie, closure of fetal shunts) for pulmonary gas exchange may form the basis of a new therapeutic platform, allowing improved outcomes for

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AJOG at a Glance

Why was this study conducted?

The study was conducted to determine the ability of a refined artificial placenta-based life support platform to maintain extremely preterm ovine fetuses 600−700 g in weight.

Key findings

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

What does this add to what is known?

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 (600−700 g). 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.

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, 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 and further extended that time to 336 hours in a subsequent set of unpublished experiments.[7-10] Other investigators have reported the successful application of artificial placenta−based therapies for up to 669 hours with similar concepts and extracorporeal gas exchange systems.[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 because of the fact that studies to date have used 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 gestational age [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.[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 birthweight infants.[2,6,15]

The etiology is multifactorial, but key contributing factors include ventilation of the immature lung, which leads to chronic lung injury,[16-20] immature vascular structure; cardiovascular and cerebrovascular autoregulatory systems, which result in higher incidence of 1 or more of such disabilities as intraventricular hemorrhage (IVH), white matter injury (WMI), and necrotizing enterocolitis[21-29]; and higher rates of life-threatening infection.[2,6,30-32] Rates of cognitive and behavioral challenges remain high, with 50−60% of extremely preterm infants displaying such disabilities.[33-35]

With the previously mentioned facts 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[36] for a period of 120 hours. The primary outcome measures were as follows: (1) maintenance of key physiological parameters; (2) absence of infection; (3) absence of brain injury; and (4) growth and cardiovascular function patterns matching that of noninstrumented, 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 hours 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/min) during the surgical procedure. Intravenous fluids (0.9% NaCl) were administered at a rate of 10 mL/kg per hour. The ewe's abdomen was clipped to expose the skin and thoroughly prepared for surgery as described previously.[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[7,37]: (1) gas exchange enhanced by lowering the priming circuit volume (from 70 mL to 50 mL) and by adapting the circuit to use only 1 high-performance membrane oxygenator; (2) using a semiclosed system incorporating a low-volume synthetic amniotic fluid bath (reduced from

approximately 35 L to 6 L); (3) frequent replacement of bath components with ultraciolet (UV)-sterilized synthetic amniotic fluid (every 6 hours); and (4) discontinuation of carotid artery and jugular vein catheters to reduce the stress on the fetus.

Study protocol

EVE group

Nine 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: (1) 1 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) 1 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 1 membranous oxygenator; (3) a second umbilical artery was catheterized while the circuit was temporally circulated with 1 umbilical artery and umbilical vein and then connected to the circuit; and (4) 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 hour shift. Twenty-four hours after commencement of the EVE therapy, normal intermittent active fetal swallowing movements, breathing movements, gross fetal body movements, and flexure and extension of the limbs were assessed at least every 6 hours. The presence of edema, ascites, pleural effusion, or bleeding was determined by ultrasound and by gross examination during necropsy after 120 hours of EVE therapy.

Lambs were continuously treated with intravenous heparin (12.5 U/kg per hour) to prevent blood coagulation, with the dose adjusted after stabilization in an attempt to maintain an activated clotting time of 180−250 seconds. Prostaglandin E_1 (40 ng/kg per minute, Tandetron; Takata Pharmaceutical, Saitama, Japan) was continuously administered after delivery. Phosphodiesterase type 3-inhibitor (0.5 μg/kg/ per minute, 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 hours) and 2 mg (estimated 2−3 mg/kg, ∼120 hours), respectively, to manage fetal critical refractory hypotension, which is often observed among extremely premature infants and associated with IVH, periventricular leucomalacia, and adverse neurodevelopmental outcomes.[39-42] Fresh frozen plasma was provided for the first hour (10 mL/kg). Midazolam was continuously administered for the first 6 hours (0.15 mg/kg per hour).

Intravenous nutrition comprised of glucose (9.5−10%), amino acids (3−3.2 g/kg per day, Pleamin-P injection; Fuso Pharmaceutical, Osaka, Japan), lipid (0.1g/kg per day, intralipid 20%; Fresenius Kabi Australia, Sydney, Australia), vitamin compounds (one eighth vial per day, Daimedin multiinjection; Nichi-Iko Pharmaceutical, Tokyo, Japan), micronutrient (0.1 mL per day, Cizanarine N injection; Nissin Pharmaceutical, Yamagata, Japan) to provide 70−75 kcal/kg per day.[14]

To prevent infection, meropenem (15 mg/kg per dose; Ranbaxy, Sydney, Australia) was administered intravenously to the fetuses every 6 hours. Intravenous fluconazole (4 mg/kg per dose, Fluconazole-Claris; AFT Pharmaceuticals Pty Ltd, Sydney, Australia) was administered to the fetuses every 24 hours. Oxygen supply to the membranous oxygenators was adjusted to maintain fetal PaO_2 between 15 and 30 mm Hg.[43] The fetuses were maintained with EVE therapy for 120 hours, followed by euthanasia with intravenous pentobarbitone (160 mg/kg per dose) for measurements of body weight, crown-rump 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. One hundred milliliters (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.[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 thawed on demand.

EVE therapy system components

Artificial placenta

The circuit was composed of 3 main parts: (1) outflow tubes; (2) membranous oxygenators; and (3) an inflow tube (all Nipro Corporation). Only 1 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−prostaglandin E_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.

Amniotic fluid

Synthetic amniotic fluid (AF) was aseptically prepared as follows: pH, 7.19 ± 0.13; sodium ion (Na+), 116 ± 4 mEq/L; chloride ion (Cl−), 113 ± 5 mEq/L; potassium ion (K^+), 6.2 ± 0.8 mEq/L; calcium ion (Ca^{2+}), 1.4 ± 0.3 mEq/L; meropenem, 167 mg/L; fluconazole 3.3 mg/L; all values for pH and electrolytes represent group mean ± SD. The AF was preheated to 39.5−40.0°C and UV treated for a minimum of 6 hours before the addition to the AF bath. The AF bath was filled with synthetic AF (6 L) and warmed constantly by 2 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 hours after the start of the EVE therapy with 30 L of new synthetic AF that had been UV treated.

Physiological, hematological, 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 (milliliters per minute) 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 were pH, base excess, pCO_2, pO_2, O_2 saturation (SO_2), O_2 content, Na^+, K^+, Ca^{2+}, Cl−, hemoglobin, lactate and glucose level (RapidPoint 500; Siemens, Munich, Germany), and activated clotting times (Haemochron Jr; Accriva Diagnostics, San Diego, CA) were measured at least every 6 hours.

Fetal umbilical arterial blood samples were collected every 24 hours following the induction of the EVE therapy. Hematological analyses, including differential leukocyte counts, and a biochemical and microbiological analysis (anaerobic and aerobic cultures) were performed by an independent clinical pathology laboratory (Vetpath, Perth, Australia).

To prevent hypoxia because of 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 per 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, Best, The Netherlands) and associated obstetrics software. For control animal measurements, ewes held in dorsal recumbency and the fetal position from the ventral aspect were confirmed by an operator.

The ultrasound beam was focused to obtain a basal 4-chamber view, 5-chamber view, left ventricular outflow tract view, right ventricular outflow tract view or 3-vessel 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 the 4-chamber view as total cardiac dimension (TCD) (Figure 1A).[46,47]

Transtricuspid and transmitral 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, ejection time, and isovolumetric relaxation time were measured for the left ventricle. Myocardiac performance index (MPI) was calculated using the formula defined as MPI = isovolumetric contraction time + isovolumetric relaxation time/ejection time 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).[54-57]

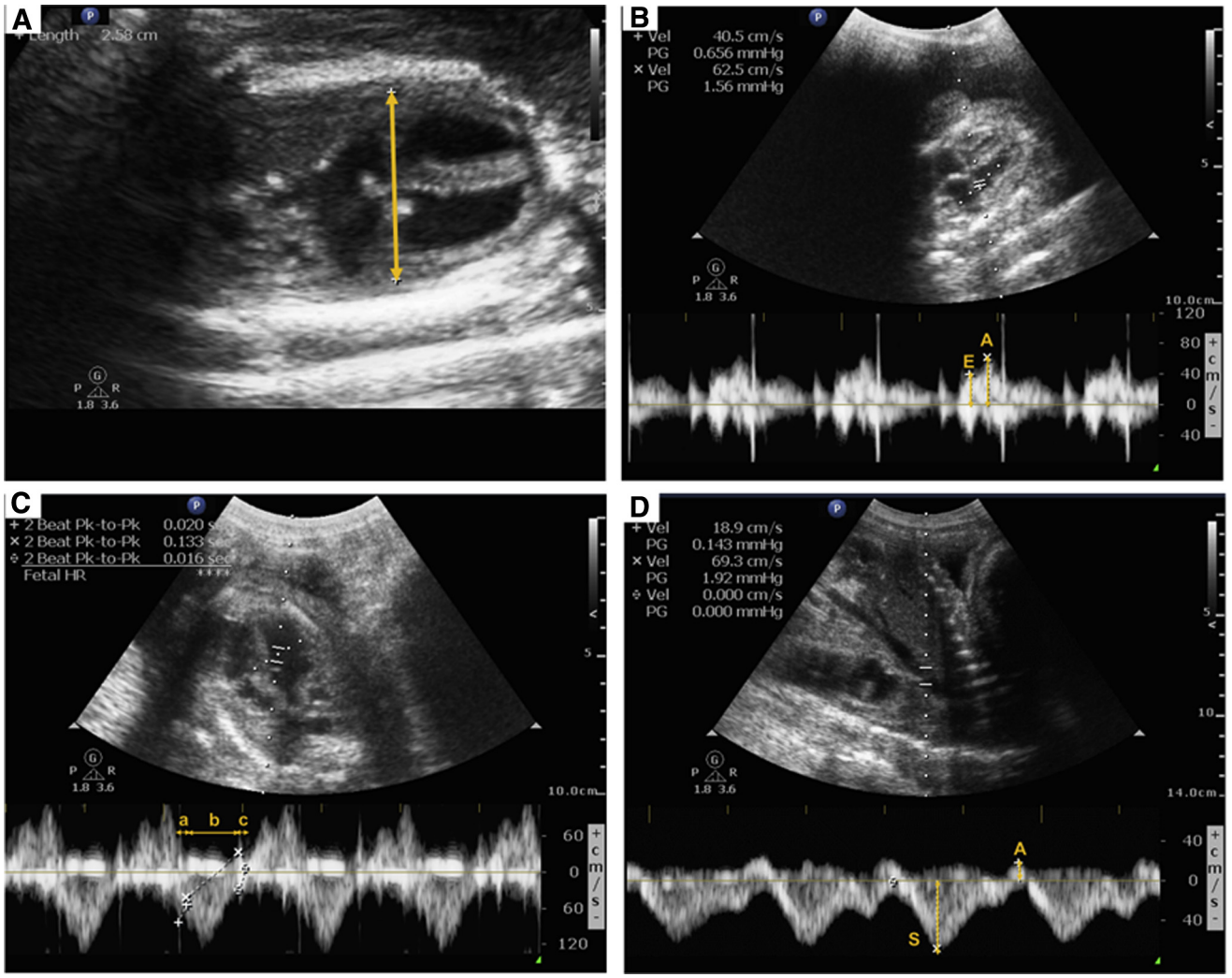
The internal diameter of the ductus arteriosus was measured at the confluence of the descending aorta. Color flow Doppler imaging was used to detect the blood flow direction through the ductus arteriosus. Blood flow from the pulmonary artery to the descending aorta was determined as right-to-left directional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

Control group

After 5 days of ultrasound assessments (as mentioned in the previous text), 9 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 and biochemical and cytokine analyses.

Reliable fetal blood gas data could not be obtained because of the euthanasia of the ewe and fetus before delivery. Therefore, the blood gas data presented

FIGURE 1
Representative images of cardiac ultrasound



A, The *arrow* indicates the 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. **B,** 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. **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. MPI was calculated as MPI = a + c/b. **D,** 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. The A/S ratio was calculated to obtain the preload index.

MPI, myocardiac performance index; *TCD*, total cardiac dimension.

Usuda et al. Successful treatment of extremely preterm ovine fetuses with ex vivo uterine environment therapy. Am J Obstet Gynecol 2019.

herein were obtained as a reference set from 13 age-matched (97 dGA ± 2 d), null-treatment fetuses previously collected as part of our ovine data bank.

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.[10] 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. One hundred microliters of each standard or sample was incubated overnight (16 hours) 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).

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 cm H_2O) for 24 hours before being washed in phosphate-buffered saline 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 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.[10,58] $CD3^+$ cells in the fetal lung were quantified by counting positively stained cells in 6 randomly selected, nonoverlapping fields at ×200 total magnification. Indirect immunofluorescent staining of positive control (right upper lobe taken from age-matched lambs with significant lung inflammation and airspace consolidation because of in utero exposure to *Escherichia coli* lipopolysaccharide) sections along with primary antibody-only and secondary antibody-only controls was used to confirm $CD3^+$ staining specificity.

Preterm brain histology

Perfusion-fixed brains 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 Larroche.[59] Briefly, each 5 mm section was visually inspected for the presence of abnormalities (petechial haemorrhage, focal coagulation necrosis). Every second 5 mm section (4 in total) was selected to be embedded in paraffin. Ten micrometer sections were then stained with 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 nonoverlapping fields (×40 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, Bedford, MA; working concentration, 1:500) and ionized calcium binding adaptor molecule 1 (IBA-1; 01919741; Wako, Richmond, VA; working concentration, 1:1000) was performed as previously published.[60,61]

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 nonoverlapping sections at ×200 total magnification.

Numbers of Oligo2-positive and IBA-1-positive 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 a group comparison of Oligo2-positive and IBA-1-positive cells with the control group. Primary antibody-only and secondary antibody-only controls were used to confirm Oligo2-positive and IBA-1-positive staining specificity.

RNA extraction and quantitative polymerase chain reaction (PCR) analysis of lung cytokine expression

Total RNA was extracted with Trizol (Life Technologies, Grand Island, NY) from 100 mg of snap-frozen fetal lung (right lower lobe) as previously described.[36,62,63] Extracted RNA was treated with Turbo-DNase (Life Technologies) in accordance with the manufacturer's instructions. RNA template was quantified using a Qubit 2.0 fluorometer (Life Technologies) using a broad-range RNA quantitation kit (Life Technologies). The 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 quantitative reverse transcription−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 the manufacturer's instructions.

Reaction cycling conditions were as follows: 15 minutes of reverse transcription at 50°C and an initial denaturation/polymerase activation at 95°C for 20 seconds, followed by 40 cycles at 95°C for 3 seconds and 60°C for 30 seconds (data acquisition phase). Target Cq values were normalized 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.[64] dCq values were used to perform statistical analyses for significant differences between intervention groups vs the control group.

Statistical analyses

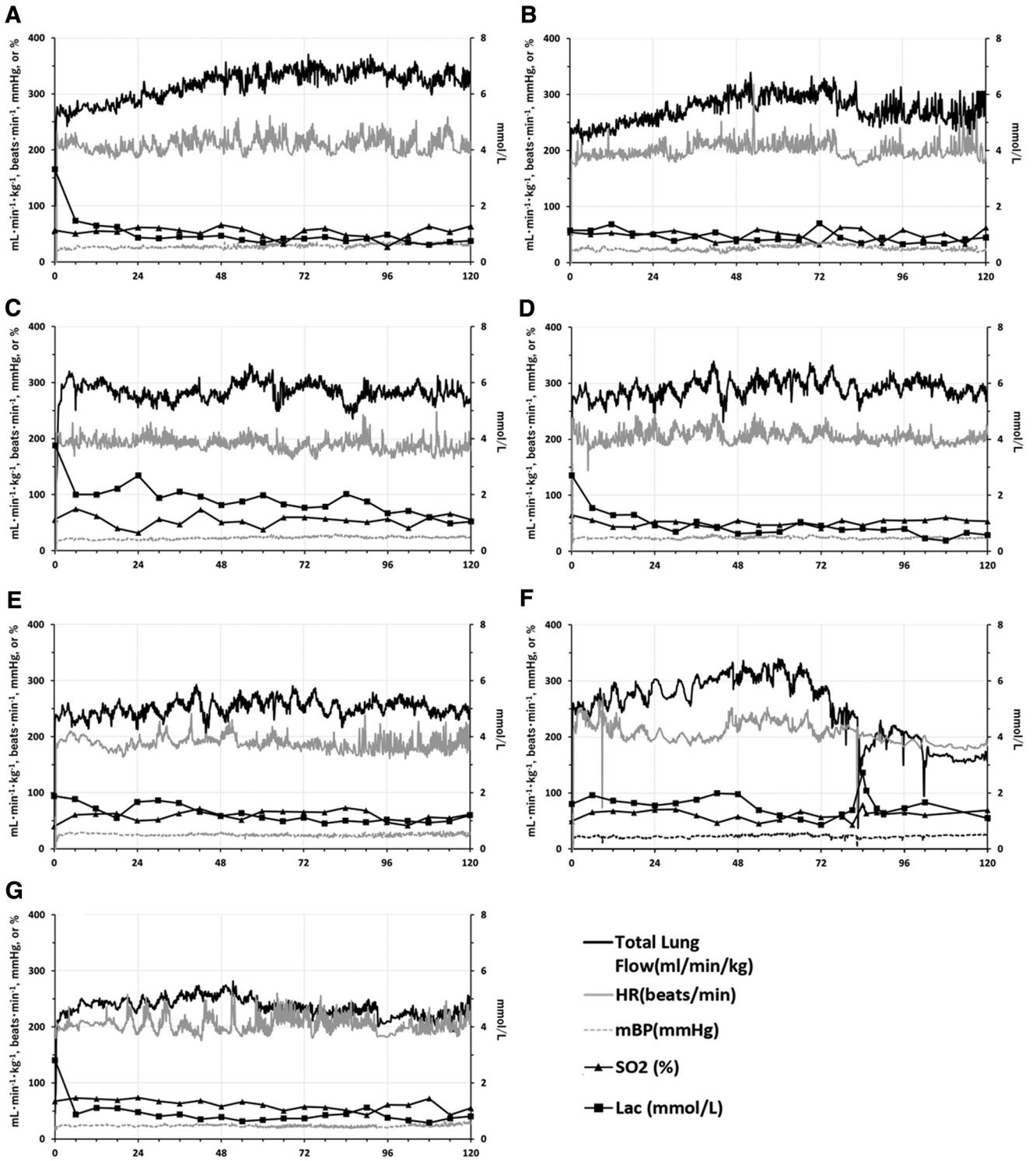
Statistical analyses were performed using IBM SPSS for Windows, version 23.0 (IBM Corporation, Armonk, NY). A χ^2 test was used to test the differences of nominal values between the 2 groups. All numerical data were tested for normality with Shapiro-Wilk tests. Extreme outliers were tested for exclusion with Smirnov-Grubbs tests. In the comparison of the 2 groups, between-group differences in the parametric data were tested for significance with Student *t* tests, whereas Mann-Whitney *U* tests were used for nonparametric data.

Results

Physiological variables

Seven of eight fetuses in the EVE therapy group completed a predetermined 120 hour experimental period. Key physiological data are presented in Figure 2. The adaptation surgery for EVE therapy was not attempted for 1 fetus because of severe growth restriction (392 g at 95 dGA, making it incompatible with the

FIGURE 2
Changes in fetal physiological and biochemical variables over time in EVE group



The horizontal axis represents the time after the induction of the EVE therapy (hours). The *black solid lines* show total oxygenator (circuit) blood flow (milliliters per kilogtam per minute); the *gray solid lines* show HR (beats per minute); the *gray dotted lines* show MAP (millimeters of mercury). The *black closed triangles* show SO_2 (percentage); the *black closed circles* show pCO_2 (Torr); the *black closed squares* show Lac (millimoles per liter). Only the blood lactate levels use the right scale bar.

HR, heart rate; *Lac*, blood lactate level; *MAP*, mean arterial pressure; *SO_2*, arterial oxygen saturation.

Usuda et al. Successful treatment of extremely preterm ovine fetuses with ex vivo uterine environment therapy. Am J Obstet Gynecol 2019.

TABLE 1
Comparison of fetal data at necropsy

Variables	Control group	EVE group	Statistical test	*P* value
Number	9	7		
Gestational age at induction of EVE therapy, d	-	94.9 ± 0.7		
Gestational age at conclusion, d	99.8 ± 0.7	99.9 ± 0.7	Mann-Whitney *U*	.812
Sex (male/female)	4/5	2/5	χ^2 test	.286
Body weight, g	1076 ± 72	1051 ± 127	Student *t* test	.620
Crown-rump length, cm	32.7 ± 1.1	32.1 ± 2.3	Student *t* test	.525
Weight-corrected crown-rump length, cm/kg	30.5 ± 1.1	30.7 ± 1.6	Student *t* test	.681
Lung weight, g	39.8 ± 3.2	39.3 ± 2.9	Student *t* test	.713
Weight-corrected lung weight, g/kg	37.0 ± 2.2	37.6 ± 3.3	Student *t* test	.673
Brain weight, g	27.3 ± 1.8	26.6 ± 1.7	Student *t* test	.397
Weight-corrected brain weight, g/kg	25.4 ± 1.3	25.4 ± 1.7	Mann-Whitney *U*	.266

Values are expressed as the group mean ± SD. $P < .05$ is considered as significant difference vs value for the control group.

Usuda et al. Successful treatment of extremely preterm ovine fetuses with ex vivo uterine environment therapy. Am J Obstet Gynecol 2019.

catheters and circuit design available for this study), and the animal was removed from analyses. Another fetus was euthanized 8 hours after the start of EVE therapy because of 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 hour study between the EVE group and the control group over the experimental period (Table 1).

Cord blood gas data had pO_2 values within the target range (15−30 mm Hg). Other items (pH, pCO_2, base excess, SO_2, O_2, O_2 content, Na^+, K^+, Ca^{2+}, Cl^-, hemoglobin, lactate level, glucose, and activated clotting time) also remained clinically acceptable and in the comparable range to the reference (Table 2).

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

TABLE 2
Blood gas data throughout experiments in the EVE group

Variables	Reference	EVE
pH	7.38 ± 0.03	7.39 ± 0.05
pCO_2 (Torr)	42.4 ± 2.9	41.7 ± 3.3
pO_2 (Torr)	25.1 ± 2.1	24.6 ± 3.6
Base excess, mmol/L	−0.2 ± 1.8	−0.3 ± 3.5
SO_2, %	65.5 ± 5.9	55.6 ± 10.0
CtO_2, mL/dL	8.9 ± 1.2	8.2 ± 1.7
Hemoglobin, g/L	93 ± 9	109 ± 14
Lactate level, mmol/L	1.7 ± 0.4	1.2 ± 0.6
Na^+, mmol/L	134 ± 3.1	138 ± 6.5
K^+, mmol/L	4.0 ± 0.3	4.2 ± 0.8
Ca^{2+}, mmol/L	1.4 ± 0.1	1.3 ± 0.1
Cl^-, mmol/L	103 ± 1.7	107 ± 7.5
Glucose, mmol/L	1.4 ± 0.2	2.1 ± 1.5
Activated clotting time, s	—	221 ± 32

Seven EVE animals were analyzed throughout the experiment. Umbilical arterial blood was collected for blood gas data every 6 hours after the start of the EVE therapy. Values are expressed as the group mean ± SD. The reference data were obtained from 13 age-matched (97 ± 2 dGA), null-treatment fetuses, which were previously collected for our ovine databank. *Ca^{2+}*, calcium ion; *Cl^-*, chloride ion; *CtO_2*, O_2 content; hemoglobin (g/dL) × 1.34 × SpO_2 (%)/100 + pO_2 × 0.003; *K^+*, potassium ion; *Na^+*, sodium ion; *SO_2*, O_2 saturation.

Usuda et al. Successful treatment of extremely preterm ovine fetuses with ex vivo uterine environment therapy. Am J Obstet Gynecol 2019.

TABLE 3
Comparison of fetal chemical data at 120 hours

Variables	Control group	EVE group	*P* value
AST, U/L	27 ± 6	68 ± 35[a]	.02 [16.2–66.7]
ALT, U/L	3 ± 3	17 ± 9[a]	.008 [4.8–22.8]
GGTP, U/L	11 ± 2	34 ± 20[a]	0.02 [5.2–41.9]
GLDH, U/L	4 ± 2	25 ± 22[a]	0.042 [1.1–41.0]
Total bilirubin, mg/dL	0.7 ± 0.2	2.3 ± 1.2[a]	0.014 [0.45–2.75]
Albumin, g/dL	1.7 ± 0.1	1.9 ± 0.1[a]	0.016 [0.04–0.38]
BUN, mg/dL	19.0 ± 3.6	39.4 ± 21.7[a]	0.025 [3.6–37.3]
Creatinine, mg/d/L	0.91 ± 0.11	0.88 ± 0.40	.883
BUN/creatinine ratio	21.7 ± 5.9	44.4 ± 5.6[a]	.001 [16.6–28.9]
Mg, mmol/L	1.2 ± 0.2	1.1 ± 0.1	.270
P, mmol/L	2.2 ± 0.3	2.0 ± 0.2	.096

Nine control animals and 7 EVE animals were analyzed. Respective values are expressed as the group mean ± SD. A Student *t* test was conducted for statistical analysis. *ALT*, alanine aminotransferase, *AST*, aspartate aminotransferase; *BUN*, blood ur ea nitrogen; *GGTP*, gamma-glutamyl transpeptidase; *GLDH*, glutamate dehydrogenase; *Mg*, magnesium; *P*, phosphates.

[a] $P < .05$ [95% confidence interval], significant difference vs value for the control group is indicated.

Usuda et al. Successful treatment of extremely preterm ovine fetuses with ex vivo uterine environment therapy. Am J Obstet Gynecol 2019.

ductus arteriosus was from right to left in each of 7 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 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 hours 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 the first 82 hours. However, the circuit flow from 1 umbilical artery deteriorated because of repositioning of the catheter tips in response to active fetal movements and frequent fetal posture change. Then circuit flow was interrupted for approximately 5 minutes with an unexpected kink of the catheters at 82 hours after EVE therapy. Circuit flow from 1 artery was restored with a 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 per hour) until the end of the experimental period. Although there was no edema, ascites, pleural effusion, or 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.

TABLE 4
Comparison of fetal cardiac ultrasound data at the conclusion

Variables	Control group	EVE group	*P* value
Total cardiac dimension, mm	24.1 ± 0.9	25.3 ± 1.2	.081
Tricuspid valve E/A ratio	0.75 ± 0.12	0.69 ± 0.06	.228
Mitral valve E/A ratio	0.76 ± 0.09	0.69 ± 0.05	.105
Myocardial performance index	0.39 ± 0.08	0.43 ± 0.06	.264
Preload index	0.32 ± 0.09	0.36 ± 0.11	.485
Dimension of ductus arteriosus, mm	5.2 ± 0.4	4.3 ± 0.8	.099
Direction of ductus arteriosus flow	Right → Left	Right → Left	—

Nine control animals and 7 EVE animals were analyzed. Values are expressed as the group mean ± SD. a Student *t* test was conducted for statistical analysis. $P < .05$ was considered as significant difference. Blood flow from the pulmonary artery to the descending aorta was determined as right-to-left directional flow.

Usuda et al. Successful treatment of extremely preterm ovine fetuses with ex vivo uterine environment therapy. Am J Obstet Gynecol 2019.

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 were edema, ascites accompanied with bladder expansion, and hydronephrosis. There were no histopathological brain injuries. No bacteria were identified from the blood culture bottle, although *Pseudomonas stutzeri* was identified in the synthetic AF culture bottles taken both from the artificial womb and the tab at the end.

EVE therapy group infection and inflammation

No bacteremia was demonstrated from the blood culture bottles in any of 7 EVE

TABLE 5
Case summary of EVE therapy group animals

Case	A	B	C	D	E	F	G
Swallowing movement	+	+	+	+	+	(24–2 H) +	+
Breathing movement	+	+	+	+	+	(24–82 H) +	+
Gross body movements	+	+	+	+	+	(24–82 H) +	+
Flexure and extension of limbs	+	+	+	+	+	(24–82 H) +	+
Edema	–	–	–	–	–	–	+
Ascites	–	–	–	–	–	–	+
Pleural effusions	–	–	–	–	–	–	–
Bleeding	–	–	–	–	–	–	–
White matter Injury	–	–	–	–	–	+	–
Blood culture	–	–	–	–	–	–	–
Culture from amniotic fluid in artificial uterus	–	–	–	–	–	–	*Pseudomonas stutzeri*
Culture from amniotic fluid in sterilized reservoir	–	–	–	–	–	–	*Pseudomonas stutzeri*
Specified issue	–	–	–	–	–	Circuit trouble because of a kink of the tip of the catheters	Hydronephrosis/ expansion of bladder

Normal intermittent active fetal swallowing movement, breathing movements, gross fetal body movements, and flexure and extension of limbs were assessed at least every 6 hours. Edema, ascites, pleural effusion, bleeding, and another specific issue were identified during necropsy after 120 hours of ex vivo uterine environment therapy. White matter injury was identified in hematoxylin and eosin–stained coronal sections. Samples for the culture bottles were collected from fetal umbilical artery, synthetic amniotic fluid in artificial womb, and sterilized tab at the end of the experimental period. +, present; –, absent.

Usuda et al. Successful treatment of extremely preterm ovine fetuses with ex vivo uterine environment therapy. Am J Obstet Gynecol 2019.

animals, although *P stutzeri* was grown from the AF culture bottles in 1 EVE animal at the end of the experimental period. There were no significant differences in the numbers of white blood corpusclse, neutrophils, lymphocytes, or monocytes between the control group and the EVE group (Figure 3A) at the conclusion of the experimental period.

Fetal plasma TNF-α protein concentrations were low and equivalent between the 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, $P = .351$, expressed as the median [interquartile range]. There were no significant differences in the relative mRNA expression of IL-1β, IL-6, IL-8, TNF-α, and MCP-1 and were identified in lung samples taken from EVE the therapy animals, compared with the control animals (Figure 3B).

No significant infiltrations of cells were identified in the pulmonary air spaces in any of 7 EVE animals that completed their protocol (Figure 4). Immunofluorescent staining for pulmonary CD3-positive cells was unremarkable, with no differences detected between control and EVE therapy samples (data not shown).

Brain histopathology

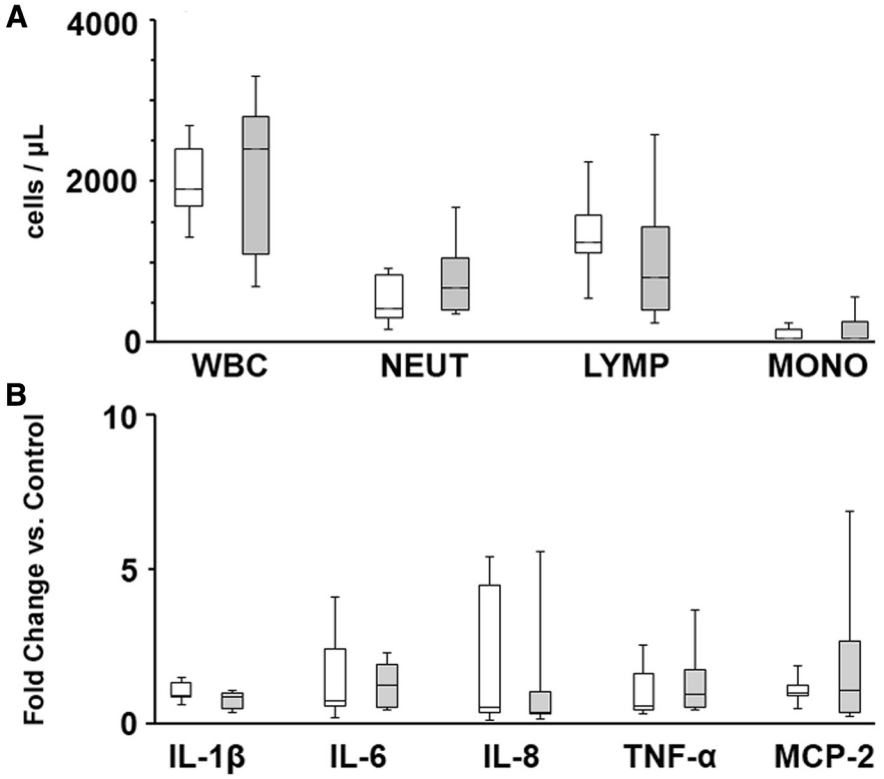
One animal (Case F) had WMI in the H&E-stained coronal sections (anterior basal ganglia level). There was no evidence of haemorrhage in any of 7 EVE animals or WMI in the remaining 6 EVE animals in gross anatomical and H&E-stained histopathological analyses of coronal brain sections. There was no significant difference in the number of either Oligo2-positive or IBA-1-positive cells in the periventricular white matter between the control group and the EVE group, apart from 1 EVE animal with periventricular WMI (Case F) (Figure 5).

Comment

The primary finding of this study is that EVE therapy allowed a 120 hour period of survival in a group of 7 extremely preterm lambs (Figure 2 and 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 1 case of circuit failure in our study, animals had otherwise stable maintenance from a hemodynamic viewpoint (Figure 2 and Tables 1–3 and 5) without any difference in cardiac ultrasound parameters between the control group and the EVE group (Table 4).

All animals were free of bacteremia, and no systemic inflammatory changes were detected (Figures 3 and 4 and Table 5 and 6). Although a treatment time of 120 hours may be limited in

FIGURE 3
Differential cell counts (blood) and cytokine mRNA expression (lung)



A, WBC. Nine control animals and 7 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 a Student *t* test with a value of $P < .05$ accepted as significant. **B,** Nine control animals and 7 EVE animals were analyzed. Relative fold changes in cytokine (interleukin-1β, interleukin-16, interleukin-1 8, tumor necrosis factor-α, and monocyte chemoattractant protein 1) mRNA expression between the control group and the EVE group in lung tissue samples. Star indicates outliners. 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 the *gray box* indicates the EVE group. Respective differences of values were tested for significance using a Mann-Whitney *U* test with a value of $P < .05$ accepted as significant.

EVE, ex vivo uterine environment; *mRNA*, messenger RNA; *WBC*, white blood corpuscle.

Usuda et al. Successful treatment of extremely preterm ovine fetuses with ex vivo uterine environment therapy. Am J Obstet Gynecol 2019.

terms of clinical use, the data from this study show 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] First, 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.

Second, 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), although total lung flow volume was maintained within or partially above the cited normal range (150−250 mL/kg per minute).[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.

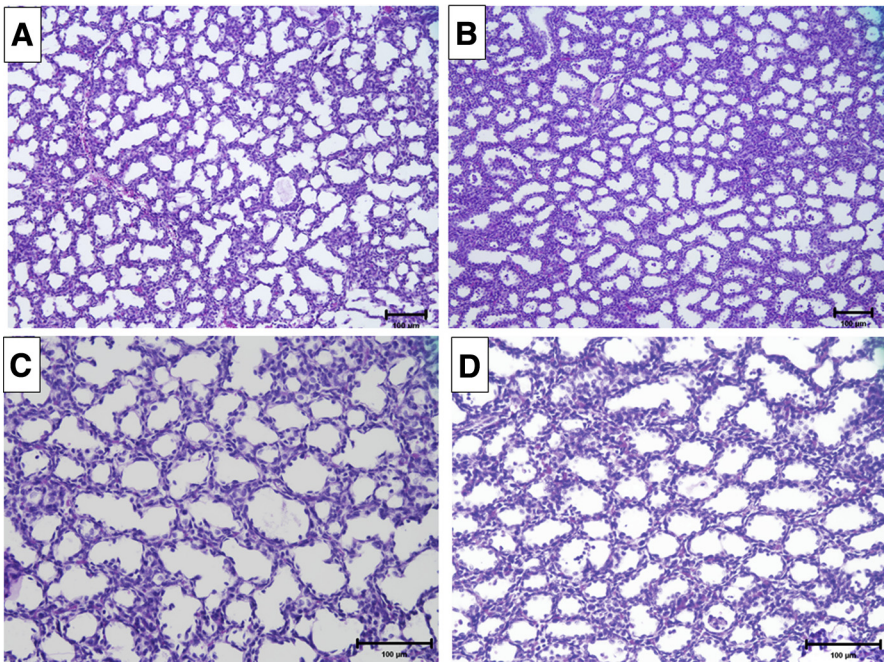
It was demonstrated that the EVE group animals were equivalent to the control group animals in fetal weight, crown-rump length, and lung and brain weights. Thus, 120 hours of EVE maintenance did not cause obvious growth restriction compared with the control animals, although a treatment time of 120 hours may be too short to examine fetal growth adequately (Table1).

Ultrasound data

The main aim of these ultrasound measurements was the assessment of cardiac disfunction 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 the 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.[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.[69] For cardiac dysfunction accompanied with intrauterine growth restriction, an increase E/A ratio is likely to be observed.[70-72] In this study, the E/A ratio in both of the control animals and the EVE animas was below 1, and the EVE animals were equivalent to the control group animals

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). **A and C,** Control tissue. **B and D,** EVE therapy group. Images are representative of H&E staining of fetal lung visualized at ×100 (panels **A and B**) and ×200 (panels **C and D**) total magnification. All scale bars indicate 100 μm.

EVE, ex vivo uterine environment; *H&E*, hematoxylyn and eosin.

Usuda et al. Successful treatment of extremely preterm ovine fetuses with ex vivo uterine environment therapy. Am J Obstet Gynecol 2019.

in both tricuspid valve and mitral valve E/A ratio.

As part of a fetal evaluation, MPI is considered a reliable early marker of fetal cardiac dysfunction. It likely represents initial stages of cardiac adaptation to different perinatal insults.[50-52] MPI data from the EVE group animals were equivalent to the control group animals and both within similar ranges (0.28−0.44) to human data.[53]

There is also an association between changes in circulation and changes in the inferior vena cava flow pattern. Increased reverse flow in the inferior vena cava during atrial contraction has been reported to suggest cardiac dysfunction.[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 the time of the 120 hour experimental period.

Brain injury

Gross anatomical observation and assessment of H&E-stained brain sections identified the presence of pathological lesions in only 1 of 7 EVE group animals (Case F). In Case F, periventricular WMI was identified in 1 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.[75] Thus, further analysis for ischemic change at the periventricular area was conducted to compare the remaining EVE animals in which periventricular WMI was not identified by H&E stain with the control animals.

Reportedl,y Oligo2-positive cells are likely to decrease in WMI; thus, we used Olig2 to identify oligodendrocyte lineage cells[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 toward WMI. Therefore, activated microglia are likely to increase in WMI, and IBA-1 was used to identify activated microglia as described previously.[60,77,78] However, the result of immunostaining demonstrated that there was no significant difference in the number of Oligo2-positive and IBA-1-positive 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 hours 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.

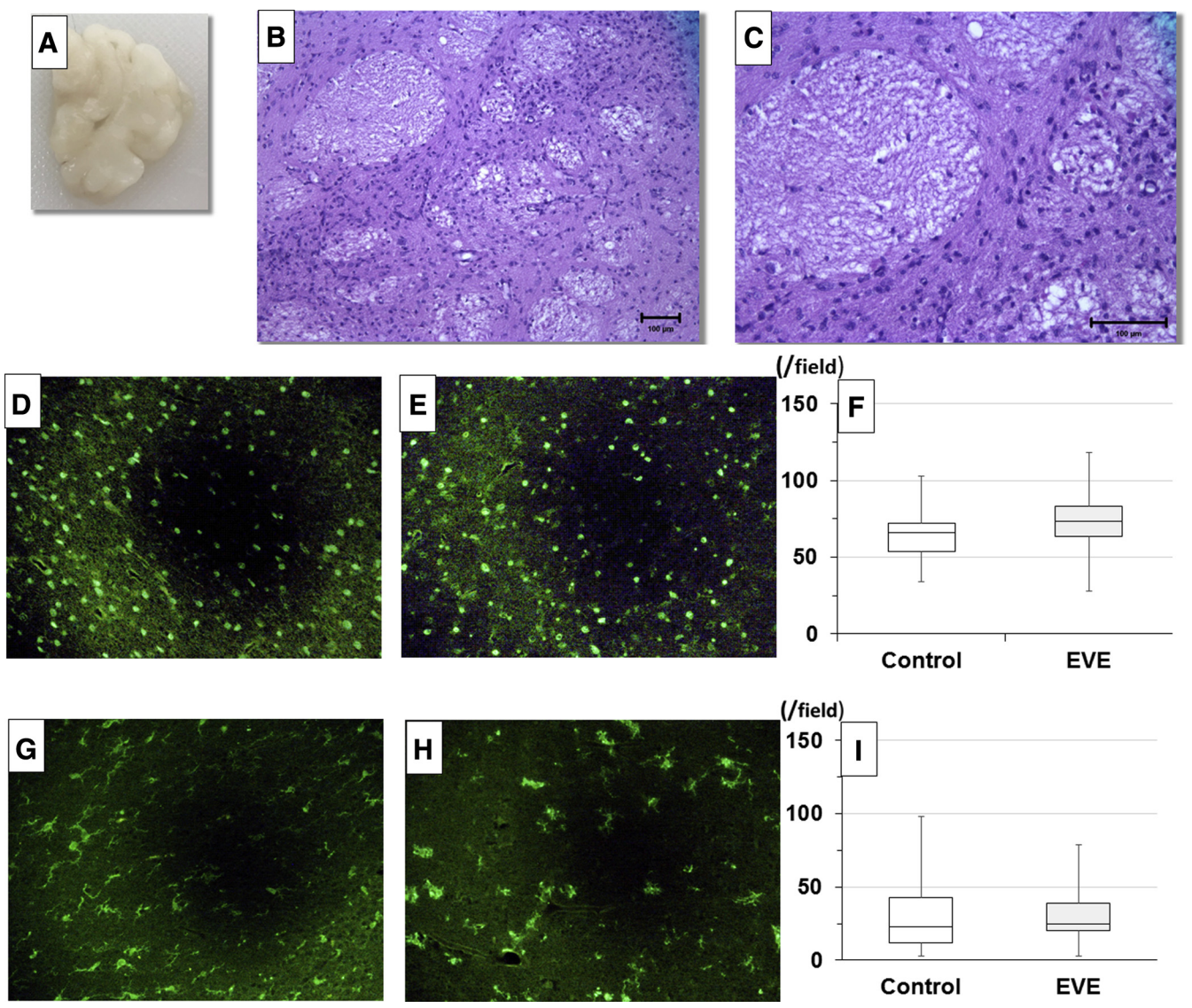
Success rate

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 hours (mortality of <28 weeks GA is overall approximately 30% of total deliveries).[6] Thus, the survival rate over the first 120 hours 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, because this was the first attempt to adapt extremely early gestational fetuses to an artificial

FIGURE 5
Representative images of brain histology



Seven EVE animals (cases A—G) were analyzed with H&E stain. Six fields from 4 5 mm serial sections were assessed for each animal. **A,** Representative gross appearance of coronal section. Representative image of white matter injury from case F identified in investigation with H&E staining shows necrosis and cellular infiltration. Images are inspected at ×100 total magnification (panel **B**) and ×200 magnification (panel **C**). Scale bar represents 100 μm. Nine control animals and 6 EVE animals (cases A—E and G) without periventricular WMI identified by H&E staining were analyzed for immunofluorescent staining (Oligo2 and IBA-1). Three fields in the periventricular area from 2 5 mm serial sections were assessed for each animal. Representative images of Oligo2-positive cells are from the control group (panel **D**) and the EVE group (panel **E**). Representative images of IBA-1-positive cells are from the control group (panel **G**) and the EVE group (panel **H**). Images are inspected at 2×00 total magnification. Comparison of the number of Oligo2-positive (panel **F**) and IBA-1; cells (panel **I**) between the control group and the EVE group without WMI were identified in the 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 the EVE group. Respective differences of values were tested for significance using a Mann-Whitney *U* test, with a value of $P < .05$ accepted as significant.

EVE, ex vivo uterine environment; *H&E*, hematoxylyn and eosin; *IBA-1*, ionized calcium binding adaptor molecule 1; *WMI*, white matter injury.

Usuda et al. Successful treatment of extremely preterm ovine fetuses with ex vivo uterine environment therapy. Am J Obstet Gynecol 2019.

placenta system, we elected to limit the 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 that affected fetal survival and well-being (including those associated with brain

TABLE 6
Success rate over time in the EVE group

Variables	Total number	Successful number	Probability, %
Survival rate	8	7	87.5
Completion rate without infection among surviving animal	7	7	100
Completion rate without brain injury among surviving animal	7	6	85.7
Completion rate without infection or brain injury among surviving animal	7	6	85.7
Survival rate without infection or brain injury	8	6	75.0

Infection was determined by microbial culture, differential cell counts, and lung histopathology. Brain injury was determined by the presence of intraventricular hemorrhage and white matter injury, identified in gross anatomical observation and historical assessment of the brain sections.

Usuda et al. Successful treatment of extremely preterm ovine fetuses with ex vivo uterine environment therapy. Am J Obstet Gynecol 2019.

injury and infection) occurred in the acute phase of the study, within the first 120 hours.

Although we have assessed a range of key physiological and hematological variables in determining the fetal well-being over the 120 hour 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 the clinical utility of this concept for extremely preterm infants.

An additional limitation of importance is uncertain hydronephrosis with bladder expansion (Figure 2G and Table 5). An obvious cause such as obstruction could not be clarified at the necropsy. Although the elimination half-life of midazolam causes urinary retention in preterm infants,[79] the active movement observed by the fetuses after midazolam cessation (6 hours) may indicate side effects of the midazolam, such as urinary retention, are unlikely. Accidental ligation of urachus when umbilical vessels were tied off during catheterization might cause expansion of the bladder and hydro nephrosis.[80,81] This may represent an animal limitation and warrants further investigation because 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 2 cases of adverse outcomes (1 fetal death and 1 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 (ie, funisitis).

In the present study, we administered hydrocortisone to manage refractory hypotension because of preliminary findings that untreated fetuses not be maintained within normal physiological variables for more than 24 hours after induction of the EVE therapy because of refractory (to continuous volume load; 20 mL/kg per hour and inotropic drug; dopamine 10 μg/kg per minute) hypotension, eventually resulting in circuit failure and fetal death. Hypotension is reported to occur in approximately 20−45% of the preterm infants,[82,83] and its prevalence is inversely related to GA.[84] About 25% of the ELBW infants with hypotension do not respond to the treatment with either volume and inotropic drug and requires hydrocortisone, independent of serum cortisol concentrations, to normalize blood pressure.[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.[39]

Our hydrocortisone maintenance assisted us in achieving 120 hours of survival time for 7 in 8 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.[87-89] Evaluation of long-term effects on the fetus because of 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, gamma-glutamyl transpeptidase, and lactate dehydrogenase compared with the control animals, which generally indicates liver disfunction, although not clinically severe.

The EVE group animals also had a BUN increase without significant creatinine increase compared with 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[90] have to be taken into consideration as the cause because it is accompanied with an 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 disfunction in premature babies.[91,92]

Although modest doses of amino acids (3 g for a total 70−75 kcal/kg for 95 dGA) for 120 hours were administered in this study, compared with the previously described dose (6 g for a total 70 kcal for 105−111 dGA) for 2−4 weeks, which did not cause any elevation of fetal plasma AST (27 U/L), ALT (3 U/L), and BUN (24 mg/dL),[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, although the appropriate administration of amino acids and total energy is still controversial, even in the human clinical field.[92]

Continuous administration of high-caloric nutrition causes hyperglycemia, however, which requires intravenous insulin administration to be administered as described previously.[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.[93,94] Pulsatile administration of high-caloric nutrition may offer a solution to this problem.[95] On the other hand, reducing amino acid dose may cause growth restriction.[92] Thus, we are still in the process of determining appropriate nutrition. The effect of long-term 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 hour 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. ■

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