Video Article

Instrumentation of Near-term Fetal Sheep for Multivariate Chronic Non-anesthetized Recordings

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Abstract

The chronically instrumented pregnant sheep has been used as a model of human fetal development and responses to pathophysiologic stimuli such as endotoxins, bacteria, umbilical cord occlusions, hypoxia and various pharmacological treatments. The life-saving clinical practices of glucocorticoid treatment in fetuses at risk of premature birth and the therapeutic hypothermia have been developed in this model. This is due to the unique amenability of the non-anesthetized fetal sheep to the surgical placement and maintenance of catheters and electrodes, allowing repetitive blood sampling, substance injection, recording of bioelectrical activity, application of electric stimulation and *in vivo* organ imaging. Here we describe the surgical instrumentation procedure required to achieve a stable chronically instrumented non-anesthetized fetal sheep model including characterization of the post-operative recovery from blood gas, metabolic and inflammation standpoints.

Video Link

The video component of this article can be found at http://www.jove.com/video/52581/

Introduction

A variety of animal models exist for the study of both normal and compromised pregnancies, including laboratory rodents, non-human primates and domestic ruminants.[1,2,3,4,5] The chronically instrumented pregnant sheep has been used extensively for 50 years as a model of human fetal development and responses to pathophysiologic stimuli such as lipopolysaccharide (LPS).[6-10] The lesions following LPS exposure mimic exactly what is seen in preterm infants with periventricular leukomalacia, which is due to a similar maturational profile of both species.[11, 12]

Other pregnancy complications have also been studied in great detail such as the discovery that antenatal glucocorticoids promote lung development [13-15] and understanding the impact of intrauterine growth restriction (IUGR) on the fetus [16,17].

The extensive use of the fetal sheep model is due to the unique amenability of the non-anesthetized fetal sheep to the surgical placement and maintenance of catheters and electrodes, allowing repetitive blood sampling, recording of bioelectrical activity, application of electric stimulation and *in vivo* brain imaging.[18] Telemetry is also possible, although less frequently used yet due to the higher sophistication to set up as well as the initial and maintenance cost.[19]

Moreover, the fetal sheep model is very versatile as many variations of instrumentation are possible depending on the measures of interest. For example, it is possible to record over days to weeks multivariate signals in real time such as fetal breathing movements, electrical brain activity, cardiovascular responses, electrocardiogram, regional blood flow to a range of organs using flow probes or microspheres, etc. Thanks to this versatility, a wide range of studies have been conducted including the development of the cardiovascular system[20,21], hypothalamo-pituitary-adrenal (HPA) axis[22], brain development[23] and sleep states development in particular[24], effects of hypoxia/asphyxia[25], therapeutic hypothermia [26], inflammation[6-11], combination of both[27], glucocorticoids[28,29], anti-depressants[30], broncho-pulmonary dysplasia (BPD) [31,32], fetal programming[33,34,35,36,37,38,39] or development of novel fetal monitoring modalities prior and during labor to name but a few areas of investigation.[40,41,42,43]

The overall goal of the method presented is to show this versatile model's basic implementation. It allows establishing a wide variety of acute and chronic experimental protocols studying fetal physiology and pathophysiology on the integrative, organ, cellular and molecular levels.

Protocol

Animal care followed the guidelines of the Canadian Council on Animal Care and the approval by the Université de Montréal Council on Animal Care (protocol #10-Rech-1560). Detailed information on materials and methods used is provided in the Table 1.

1. Anesthesia

1. Insert a single-lumen catheter into a jugular vein.
2. Sedate the ewe using acepromazine (Atravet 10 mg/mL) 2 mg intravenously approximately 30 min prior to the induction of anesthesia in order to reduce stress associated with the procedure which in turn reduces levels of cortisol.
3. Administer diazepam (Diazepam 5 mg/mL) 20 mg, ketamine (Ketalar 100 mg/mL) 4-5 mg/kg and propofol (Propofol 10 mg/mL) 0.5 to 1 mg/kg intravenously to induce general anesthesia.
4. Insert an airway exchange catheter into the trachea using a laryngoscope with a Wisconsin type blade (Extra-long 350 mm Left-Handed Blade) to aid with the intubation. Slide the silicon endotracheal tube (9 to 12 mm Inner Diameter) off the airway exchange catheter and into the trachea. This technique facilitates the intubation process. Inflate the cuff of the endotracheal tube carefully to avoid pressure-induced ulceration of the trachea and fixate the tube to the head of the ewe.
5. Connect the endotracheal tube to the respiratory circuit of the anesthetic machine and begin mechanical ventilation immediately. Adjust ventilator settings to maintain a P_aCO_2 within normal limits of 35 to 45 mmHg.
6. Insert a catheter into the auricular artery (22 to 20 G; 1 in [0.9 x 25 mm] to 1.16 in [1.1 x 30 mm]) and connect to non-compliant tubing to monitor direct arterial blood pressure.
7. Use a multi-parameter physiologic monitor to record the electrocardiogram, direct arterial blood pressure, oxygen saturation (SpO_2), capnography ($P_{ET}CO_2$), and temperature every 5 min. Transfer all physiologic data via a serial cable to a central physiologic data-collecting computer. Maintain normal body temperature using a circulating water blanket.
8. Administer a balanced poly-ionic solution at 10 mL/kg for the first hour of general anesthesia and then reduce to 5 mL/kg/h.
9. Administer trimethoprim-sulfadoxine 5 mg/kg IV to the ewe just prior to the skin incision as antibiotic prophylaxis.
10. Use standard aseptic techniques with all surgical manipulations of the ewe and fetus.
11. Barrier nurse the ewes at all times. This includes also the non-surgical personnel. This will minimize the enzootic potential for *e.g., Coxiella burnetii.* Use gloves and masks (N-95 type) at all times.

2. Overview of the Surgical Procedure

1. Make a 20 cm midline incision through the lower abdominal wall immediately cranial to the udder through the linea alba to minimise abdominal muscle damage.
    1. Retract the greater omentum cranially and the uterine horns are palpated manually. Palpate each horn from the body of the uterus to the tip of the horn noticing the number of fetus and their size. If there is more than one fetus, choose the larger fetus by evaluating manually the head size and the width between the orbits.
    2. Hold the head of the chosen fetus firmly through the uterus partially exteriorized. Perform a 10 cm hysterotomy on the large curvature with Metzenbaum scissors. Put immediately over the head a non-latex sterile surgical glove filled with sterile saline as if it were a hand. Alternatively, use moistened 4x4s to keep the fetal head moist. Secure the uterus to the abdominal wall.
        1. Exteriorize the right and left thoracic limbs and pull the fetus gently out of the uterus up to the xyphoid process.
    3. Insert polyvinyl catheters into the right and left brachial vein and arteries using a standard cut-down technique. Insert another polyvinyl catheter into the amniotic cavity by fixating its' end to the sternum of the fetus.
        1. Use sterile catheters only. We recommend gas sterilization in your standard facility. Attach each catheter to a needle and the needle to a double stopcock to allow for later blood sampling or pressure monitoring.
    4. Suture stainless steel electrodes to the manubrium, xiphoid process and to each point of the shoulder to monitor the electrocardiogram (ECG).
    5. Return the fetus to the uterus. All catheters and electrodes exit via a small stab incision in the left flank.
    6. Close the laparotomy incision using a three layer closure. Suture the abdominal wall with a synthetic absorbable monofilament suture USP 2 in a simple continuous fashion. Close the subcutaneous space with a synthetic absorbable braided suture USP 0 in a simple continuous fashion. Use surgical stainless steel staples to close the skin incision.

2. Detailed description of the surgical procedure
    1. Remove wool by shaving the xyphoid process to the mammary gland and along the fold of the flank on each side with a blade # 40. Clean the ventral abdomen then thoroughly with a 4% chlorhexidine gluconate and a soft brush for 3 min.
        1. Perform a standard sterile scrub with chlorhexidine gluconate 4% starting from the center of the abdomen and progressing in a centrifugal fashion for 3 min. Pour sterile saline on the abdomen to remove the disinfectant soap. For the final step of the surgical preparation, perform three alternate passages of Chlorhexidine gluconate solution 2% and isopropyl alcohol 70%.
    2. Ensure the depth of anesthesia is adequate prior to incision. Make a standard laparotomy incision from the umbilicus to just cranial to the udder through the linea alba to minimise abdominal muscle damage.
    3. Insert a long sponge forceps in the abdomen along the left abdominal wall up to the planned exit site for catheters in the paracostal region.
        1. Push the tips of the forceps against the wall until an assistant can locate it and confirm the appropriate site. Open slightly (1 cm) the jaws of the forceps and let the assistant make a 2 cm full-thickness stab incision.

2. Exteriorize the tips of the forceps through the incision, open again, and gently grasp the catheters with the forceps that is finally pulled out of the abdomen through the ventral abdominal incision. Of note, some groups implant the catheters and then exteriorize them. This has the drawback that intrasurgical fetal ECG monitoring is not possible.

4. Palpate the uterus to determine fetal position and numbers. Determine the largest fetus using the inter-aural distance. Incise the uterine wall on the large curvature over the dorsum of the head, avoiding the cotyledons.
    1. Insert a blunt-ended cannula through the placental membranes to obtain an amniotic fluid sample free from hemorrhage. Incise the placental membranes using scissors.

5. Exteriorize the cranial half of the fetus through this incision. Place a sterile non latex surgical glove filled with sterile saline at 37 °C over the fetus head to help maintain normothermia.
    1. During removal of the fetal upper body from the uterus, have the assistant hold the Babcock's up to prevent the loss of amniotic fluid. Then, again using Babcock forceps, clamp the fetal membranes and uterine wall to the skin to prevent abdominal contamination with amniotic fluid.
    2. Expose only the parts of the fetal body that need to be instrumented and keep the remaining parts inside the uterus or covered by moist and warm (37 °C) sterile cloths, respectively.

6. Abduct both thoracic limbs to facilitate the exposure to the brachial artery and vein bilaterally. Incise along the medial aspect of both antebrachium and carefully dissect around the brachial artery and vein.
7. Insert polyvinyl catheters into the right and left brachial arteries and the left brachial vein using a standard cut-down technique.
    1. Free the vessel to be catheterized from adjacent tissue over 1 cm. Ligate the distal portion of the vessel with a braided synthetic absorbable USP 2-0 suture. Preplace a ligature at the proximal aspect of the vessel but keep it untied.
        1. Using Castroviejo scissors, cut the vessel transversally to approximately 30% of its diameter. Stop the blood flow partially by pulling on the proximal suture. Insert the catheter in a proximal direction.

    2. Insert the polyvinyl catheter up to 8 cm proximally or until resistance is detected and then pull back slightly. Temporally secure the proximal aspect of the catheter using a vascular clamp. Place another suture around both the proximal and distal aspect of the catheter. An assistant is continuously aspirating and flushing the catheter to ensure patency of the catheter.
    3. Close the fetal skin using a USP 2-0 braided synthetic absorbable suture, with a continuous suture pattern.

8. To the right and left shoulder, manubrium and xyphoid process, fixate an insulated stainless steel electrode to facilitate the monitoring of the fetal ECG.
9. Suture the amniotic pressure and sampling catheter to the sternum. This catheter is fenestrated at its extremity.
10. Secure all the catheters on the dorsum of the fetus using a USP 2-0 braided synthetic absorbable suture material.
11. Prior to the replacement of the fetus back into the uterus, administer clenbuterol 30 µg IV slowly over 15 min to avoid hypotension and to provide uterine relaxation.
12. Suture the fetal membranes using USP 4-0 braided synthetic absorbable suture material with a continuous pattern. Incorporate only one catheter or electrode at a time into the closure to ensure a tight closure. Use a double layer Cushing pattern respecting Halsted principles to close the uterine muscular layer using a USP 0 braided synthetic absorbable suture material. Bury the surgical knots carefully.
13. Using a purse-string suture pattern, secure all the catheters and ECG cables as they exit the left paracostal incision.
14. Using a USP 2 monofilament synthetic absorbable suture material secure the linea alba with a continuous pattern. Close the subcutaneous tissues using USP 2-0 braided synthetic absorbable suture material with a continuous pattern. Secure the skin layer with surgical staples.
15. Administer 250 mg of ampicillin intravenously and again via the amniotic catheter into the amniotic cavity. Replace lost amniotic fluids with warm saline.
16. Place all the exteriorized catheters and ECG electrodes into a place bag to maintain sterility. Place a stockinette around the torso of the ewe to secure all the catheters and electrodes to the body of the ewe.
17. Stop general anesthesia, and extubate the ewe once laryngeal reflexes have returned to normal.
18. Return the ewe to a metabolic cage once she is stable following general anesthesia. The ewe will reside in the metabolic cage for the duration of the experiment. The ewe should be able to stand, lie down and eat *ad libitum* while monitoring the non-anesthetized fetus without sedating the mother.
19. For the following three days, administer antibiotics prophylactically to the ewe (Trimethoprim sulfadoxine 5 mg/kg) and fetus (250 mg of ampicillin intravenously and again via the amniotic catheter).
20. Evaluate the metabolic status of both ewe and fetus using blood gas analyses.
21. Flush all catheters with the minimal volume of heparinized saline possible. Caution – do not exceed the daily dose of heparin and fluids permissible to the fetus. It is possible to fluid overload the fetus. Flush slowly once a day ~5 mL NaCl per line after antibiotic prophylaxis.

3. Data recording and analysis
    1. During surgery, optionally record the maternal and fetal ECG and heart rates as well as maternal arterial blood pressure and airway pressure (Paw) continuously (**Figure 1**). Use the Life Window Monitor to acquire all maternal data except ECG. Feed these data into analog-digital converter along with fetal and maternal ECG signals; pass maternal and fetal ECG first into 1901 pre-amplifier. Record and display all data in manufacturer's software.
    2. Take a 1 mL arterial sample simultaneously from the ewe and fetus for arterial blood gas analysis, lactate, glucose and base excess determination (in plasma) at the begin of the fetal surgery (immediately after inserting the first arterial catheter) and after closing the uterus.
    3. During postoperative recovery, take a 3 mL fetal blood sample to measure the IL-6 and TNF-α inflammatory profiles. Centrifuge the plasma at 4 °C (4 min, 4,000 x g), decant and store the plasma at -80 °C for subsequent ELISA testing.

Note: For the purpose of the reported representative results, six days after surgery the animals were sacrificed using intravenous injection of 20 mL sodium pentobarbital. Fetal growth was assessed by body, brain, liver and maternal weights. The duration of the experimental period will obviously vary depending on the design chosen for the particular research question and can reach ~6 weeks.

4. Cytokine analyses (optional step)
    1. Determine cytokine concentrations (IL-6, TNF-α) in plasma using an ovine-specific sandwich ELISA. Pre-coat mouse anti-sheep monoclonal antibodies (capture antibody IL-6) or mouse anti-bovine monoclonal antibody (TNF-α) at a concentration 4 µg/ml on ELISA plate at 4 °C for O/N, after 3 times wash with washing buffer (0.05% Tween 20 in PBS, PBST).
    2. Block the plates for 1 h with 1% BSA in PBST. Wash the plates with washing buffer 3 times.
    3. Use recombinant sheep proteins (IL-6, TNF-α) as ELISA standard, prepare a serial dilutions range from standard 1 of 2,000 ng/ml to standard 7 of 31.25 pg/ml.
    4. Load 50 µl of serial diluted protein standards and samples per well and incubate for 2 hr at RT, wash the plates 3 times. Run all standards and samples in duplicate.
    5. Apply 50 µl of rabbit anti-sheep polyclonal antibodies (detection antibody IL-6) or rabbit anti-bovine polyclonal antibody (TNF-α) at a dilution of 1:250 in wells and incubate for 30 min at RT. Wash the plates with washing buffer 5 times.
    6. Add 50 µl of the goat anti-rabbit IgG-HRP conjugated (dilution 1:5,000) for 30 min.
    7. Incubate with 50 µl of TMB substrate solution per well.
    8. Stop colour development reaction at desired time with 25 µl of 2 N sulphuric acid.
    9. Read the plates on ELISA plate reader at 450 nm, with a 570 nm wavelength correction.
    Note: In our assays, the sensitivity of IL-6 ELISA was 16 pg/ml, the sensitivity of TNF-α ELISA was 13.9 pg/ml, respectively. For all assays, the intra-assay and inter-assay coefficients of variance was <5% and <10%, respectively.

5. Statistical analyses
Note: The exact statistical methods will depend on the research question. Here we report the methods used to test for any significant differences in the blood gases reported in Table 2.
    1. Test normal data distribution using Kolmogorov-Smirnov test followed by parametric or non-parametric tests with adjustment for multiple comparisons, as appropriate.
    2. Use K-means cluster analysis to identify the fetuses in the cohort that were spontaneously hypoxic and determine the respective pO_2 and O_2Sat values (consider pO_2 < 20 mmHg or O_2Sat < 55% as fetal hypoxia). [44-48]
    3. Use statistical software such as SPSS for the analyses.
    4. Present data as Mean±SD with statistical differences at $p<0.05$ considered significant.

Representative Results

38 pregnant time-dated ewes were instrumented at 128±2 days of gestation (dGA, ~0.88 gestation, term 145 dGA) with arterial, venous and amniotic catheters and electrocardiogram (ECG) electrodes with sterile technique under general anesthesia (both ewe and fetus). In case of twin pregnancy the larger fetus was chosen based on palpating and estimating the intertemporal diameter; alternatively, the fetus to be instrumented may be selected randomly to avoid any potential bias or both fetuses may be instrumented. The total duration of the procedure was 124±27 min. The portion of the fetal upper body to be instrumented remained outside the uterus for 92±19 min. Most of the ewes instrumented were F2 animals. Their dam was F1 (Border Leicester*Romanov) and the sire was a Hampshire ram; they were crossed as follows: Hampshire (50%) - Border Leicester (25%)- Romanov (25%) = HABLRV.

Representative maternal and fetal physiological characteristics during surgery and instrumentation are shown in **Figure 1** and were within the physiological norm for the gestational age of the fetus and maternal behavior during anesthesia.

Maternal weights averaged 75±11 kg and 21 out of 38 carried twins (*i.e.*, at a rate of 1.6±0.5). At the time of necropsy (134±3 dGA), among the instrumented fetuses singletons weighed 4,090±800 g while twins' weight was lower at 3,300±740 g (p=0.003). The weight of uninstrumented twins at 3,300±670 g was similar to the weight of the instrumented twins (p=0.78). 18 of the instrumented fetuses were female and 20 were male.

The dynamics of fetal arterial blood gases, glucose and acid-base status during surgery and post-operative recovery are reported in Table 1. We observed a gradual recovery of fetal acid-base status and a moderate deterioration of oxygenation with little change in glucose and electrolytes from post-operative days 1 to 3. Notably, on post-operative day 3, 42% of fetuses were found to be spontaneously hypoxic with arterial pO_2 11 mmHg of and O_2Sat of 28%. The normoxic cluster's fetal pO_2 was centered at 22 mmHg and O_2Sat at 56%. Twin fetuses were not more hypoxic than singleton fetuses (p=0.26).

Fetal arterial IL-6 ELISA rendered values below the sensitivity threshold of 16 pg/ml throughout the post-operative recovery period. Similarly, the TNF-α levels also remained unchanged and very low at 29 pg/ml with ~30% of the animals also showing values below the sensitivity threshold of 13.9 pg/ml throughout the post-operative recovery period.

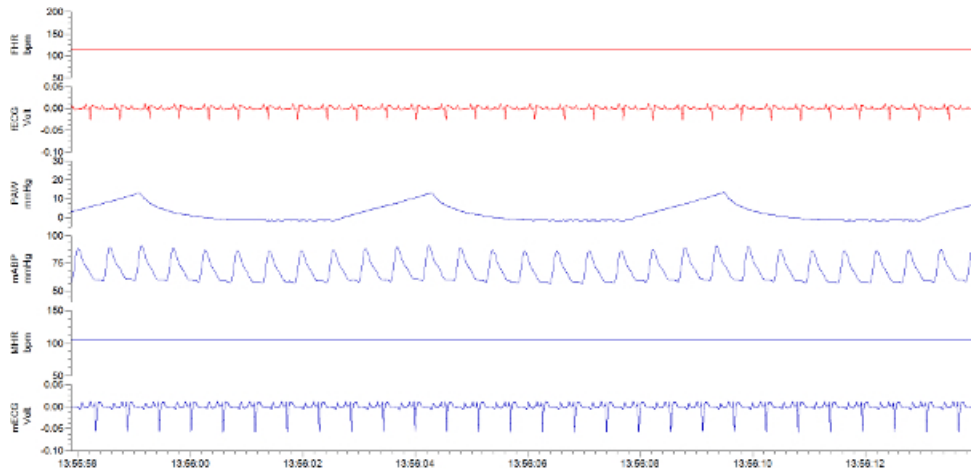

Figure 1. Intrasurgical maternal and fetal monitoring. FHR, fetal heart rate in beats per minute (bpm); fECG, fetal electrocardiogram (V); PAW, maternal positive airway pressure (mmHg); mABP, maternal arterial blood pressure (mmHg); MHR, maternal heart rate (bpm); mECG, maternal ECG (V). The X axis is the time scale in the hh:mm:ss. Please click here to view a larger version of this figure.

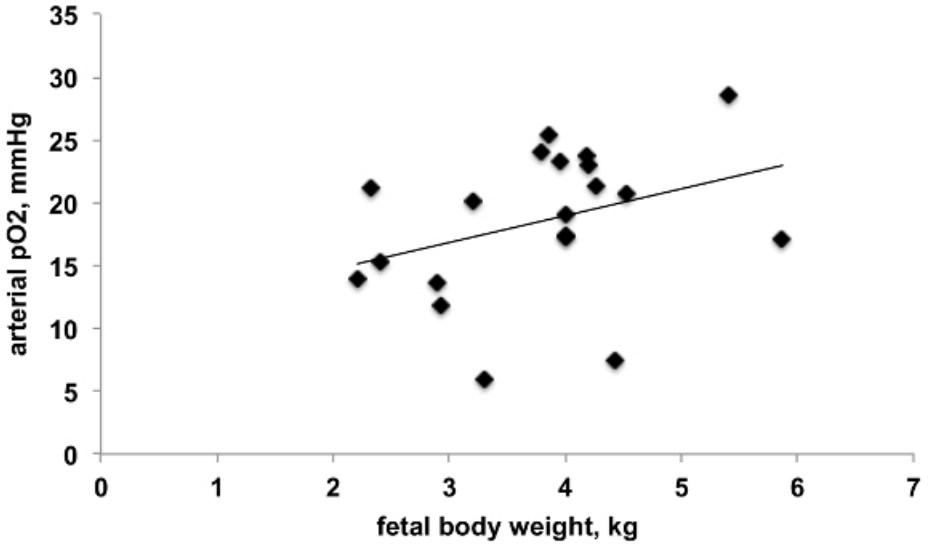

Figure 2. Spontaneous fetal hypoxia and body weight. No relationship between fetal body weight and arterial pO_2 at post-operative day 3 was detected (Spearman R=0.326, p=0.161). Please click here to view a larger version of this figure.

ACE Light source	Schott-Fostec	A20500	
Dissecting scissors	Fine Science Tools	14060 - 11	
Angled dissecting scissors	Fine Science Tools	15006 - 09	
Scalpel handle	Fine Science Tools	10003 - 12	alternative dissecting tool
Curved scalpel blades #12	Fine Science Tools	10012 - 00	alternative dissecting tool
Bone scissors	Fine Science Tools	16044 - 10	
S & T suture tying forceps	Fine Science Tools	00272 - 13	
Dumont SS forceps - angled	Fine Science Tools	11203 - 25	
Braided silk suture size 6-0	Teleflex Medical	07 - 30 - 10	
Medical Tape	transpore	3M	
Ketamine hydrochloride 100 mg/ml	Hospira	NDC 0409 - 2051 - 05	Final Does is 80 mg/kg
Tranqui Ved Injection (xylazine 100 mg/ml)	Vecdo	NDC 50989 - 234 - 11	Final Does is 10 mg/kg
Reactive orange 14	Sigma - Aldrich	R - 8254	

Ringers Solution Components			Solution is gas equilibrated with 95% O2 and 5% Co2, final pH 7.4
Sodium chloride	Sigma - Aldrich	S7653	Final Concentration: 118 mM
Potassium chloride	Fisher Scientific	P217 - 3	Final Concentration: 4.7 mM
Calcium chloride dihydrate	Fisher Scientific	C79 - 500	Final Concentration: 2.5 mM
Potassium phosphate monobasic	Fisher Scientific	P -285	Final Concentration: 1.2 mM
Magnesium sulfate	J.T. Baker	Jan-00	Final Concentration: 0.57 mM
4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES)	Fisher Scientific	BP 310 - 500	Final Concentration: 5.95 g/L
Glucose	Sigma - Aldrich	G8270	Final Concentration: 5.5 mM
LifeWindow	Digicare Biomedical Technology		
CED bioamplifier and ADC units	Cambridge Electronic Design Limited, Unit 4, Science Park, Milton Road, Cambridge CB4 0FE ENGLAND.	Bioamp: 1902; ADC: micro1401; Data acquisition software: Spike 2, V7.13	
Neurolog analog signal bioamplifier	Digitimer Ltd 37 Hydeway Welwyn Garden City Hertfordshire, AL7 3BE, England	NL108A	
ABL800Flex	Radiometer Canada; 200 Aberdeen Dr, London, ON N5V 4N2		
Eppendorf 5804R	Eppendorf Canada; 2810 Argentia Road, #2 Mississauga, Ontario, L5N 8L2		
Arrow Jugular Catheterization Set	Arrow International, Inc., 2400 Bernville Road, Reading, PA 19605 USA		
Atravet			10 mg/ml
Diazepam			5mg/ml
Ketamine	Ketalar		100 mg/ml
Propofol			10 mg/ml
SurgiVeT	Endotracheal Tubes; Smiths Medical ASD, Inc. St. Paul, MN 55112, USA		
Cook Airway Exchange Catheter with RAPI-FIT Adapters	Cook Critical Care 750, Bloomington IN 47402-0489 USA		
Dispomed Ventilator	Dispomed Ltd., 745 Nazaire-Laurin, Joliette, Quebec J6E 0L6		
BD Insyte-W	Becton Dickinson, Infusion Therapy Systems Inc., 9450 S State St, Sandy Utah 84070 USA		22 to 20 G; 1 in [0.9 x 25 mm] to 1.16 in [1.1 x 30 mm]
Edwards Lifesciences Ref: PX272 Pressure monitoring kit with TruWave Disposable Pressure			
LifeWindow LW6000	Digicare Biomedical Technology 107 Commerce Road, Boynton Beach, FL 33426-9365 USA		
Gaymar			
Babcock			
Polyvinyl catheters	SCI (Scientific Commodities Inc.)		2 meters
2-0 Vicryl			

Castroviejo scissors			
electrocardiogram (ECG)	LIFYY, Metrofunk Kabel-Union, Berlin, Germany		four copper electrodes in single sheath, 2 meters
2-O Vicryl			
3-0 Vicryl			
PDS II USP			
Trimethoprim sulfadoxine			
Ampicillin			
Stopcock	Argon Medical, Cat 041220001A		Double 4-way Stopcock with male luer lock
Needles	Tyco Healthcare 8881202389		Monoject aluminum hub blunt needles, 22Gx, 0.7mmx 38.1mm: for fetal arterial and venous catheters
Needles	Tyco Healthcare 8881202322		Monoject aluminum hub blunt needles, 16Gx, 1.6mmx38.1mm: for amniotic catheters

Table 1. Specific Reagents/Equipment.

	pH	$paCO_2$	paO_2	Hb	Hct	O_2Sat	O2Content	Glucose	Lactate	HCO_3	BE	Na	K	Ca	Cl
Uterus open	7.250 ± 0.060	57.5 ± 8.8	29.6 ± 7.3	12.1 ± 1.8	37.8 ± 6.1	71.7 ± 13.4	11.6 ± 2.5	14.7 ± 4.9	2.1 ± 0.6	24.3 ± 2.4	-1.5 ± 2.6	139.3 ± 2.6	3.5 ± 0.5	1.8 ± 0.7	108.2 ± 6.2
Uterus closed	7.256 ± 0.050	55.4 ± 11.0	29.6 ± 10.6	11.6 ± 1.5	35.9 ± 4.7	67.7 ± 14.5	10.5 ± 2.4	16.9 ± 3.9	2.1 ± 0.6	23.9 ± 3.1	-1.8 ± 3.2	139.5 ± 2.4	3.2 ± 0.6	1.6 ± 0.6	107.7 ± 6.5
Postop D1	7.356 ± 0.045	49.1 ± 5.7	21.2 ± 12.7	11.3 ± 2.4	36.6 ± 6.0	50.4 ± 15.4	7.6 ± 2.5	16.3 ± 6.3	2.0 ± 0.8	26.0 ± 3.5	1.5 ± 3.7	141.4 ± 3.7	3.9 ± 0.7	1.5 ± 0.7	104.4 ± 5.2
Postop D2	7.353 ± 0.030	51.1 ± 5.6	22.2 ± 15.1	10.2 ± 2.7	32.3 ± 9.1	45.4 ± 19.1	6.5 ± 2.8	16.8 ± 5.7	1.8 ± 0.8	26.3 ± 3.3	2.0 ± 2.4	140.3 ± 3.2	4.0 ± 0.5	1.5 ± 0.6	104.3 ± 7.5
Postop D3	7.355 ± 0.030	53.2 ± 6.1	18.4 ± 6.2	10.8 ± 1.6	33.8 ± 4.8	48.1 ± 15.7	6.9 ± 2.2	16.9 ± 4.7	1.9 ± 1.5	27.9 ± 2.5	3.3 ± 2.5	140.4 ± 4.0	4.1 ± 0.5	1.5 ± 0.6	100.7 ± 3.7

Please click here to view a larger version of this figure.

Table 2. Complete overview of fetal blood gases, metabolites and electrolytes during surgery and post-operative recovery. Fetal arterial blood pH, pCO_2 (mmHg), pO_2 (mmHg), oxygen saturation (O_2Sat %), glucose (mg/dL), lactate (mmol/L) and base excess (mmol/L) at various time points of the instrumentation and recovery periods: fetal surgery's start immediately after installing the first fetal arterial catheter (uterus open), fetal surgery's end (uterus closed), post-operative days 1 to 3. Please click here to view a larger version of this figure.

Discussion

The anesthetic and surgical procedures are presented that are required for establishing an animal model for studying fetal physiology and pathophysiology: the chronically instrumented non-anesthetized fetal sheep.

Four critical steps within the protocol should be emphasized. First, passing the catheters and electrodes through the maternal flank: it is important that this is done at once to avoid any internal organ injuries. Second, securing the uterotomy operating site prior to exteriorizing the fetus: this is crucial to prevent or minimize loss of amniotic fluid and subsequent suturing of the amniotic membrane prior to uterine closure. Third, arterial catheterization: fetal sheep arteries are about 1-2 mm in diameter and hence not difficult to catheterize for an experienced surgeon; a team of two surgeons performs best and quickest in this task which helps save time to minimize the overall length of the procedure. Fourth, careful securing and organization of all catheters and electrodes in the amniotic cavity prior to returning the fetus into the amnion and closing the uterus: this helps to avoid accidental pulling of the catheters or tearing of the ECG electrodes due to maternal or fetal movements after surgery.

Instead of the here presented approach to catheterize brachial vessels, carotid or femoral vessels can also be used. The choice depends on the overall instrumentation approach that in turn will be dictated by the study design. We recommend to minimize the time the fetus spends outside the uterus and to minimize the extent to which the fetus needs to remain outside the uterus to be instrumented. These considerations led to the choice of the vessels instrumented in the presented "minimal approach". We recommend catheterizing arteries on both sides to allow for intra-arterial blood pressure monitoring and arterial blood sampling with no mutual interference throughout the experiment. An added advantage is the fail-safe redundancy this approach introduces: in case one artery does get blocked during the experimental period, sampling and pressure monitoring are possible from the same vessel with the drawback of interrupting the monitoring when blood sampling is done.

There are three limitations, which preclude a wider adaptation of this animal model. These limitations can be addressed by some modifications suggested below. First, it is the requirement for biosafety level 2 confinements in some jurisdictions. This is due to a risk for *Coxiella burnetii* infection from pregnant sheep in humans with a weakened immune system. [49,50] Vaccination is available for the animals and exposed humans to decrease this risk [51,52] and PCR tests can be done to ensure that no positive animals are delivered to the research facility from the farm. A solution can be to combine animal vaccination with multiple PCR tests on the farm from the vaginal swabs performed prior to breeding begin, prior to delivery and then again from the amniotic fluid during the surgery. With such precautions, in some jurisdictions the use of sheep in research is not limited as it is in others. Second, the cost per animal is in the lower four-digit range, comparable to some murine knockout strains. With this in mind however, the information gain from each fetal sheep experiment compares only to non-human primates as it pertains to the extensive amount of data that can be collected, the questions that can be asked and the potential of translation to the human due to the timing of the development of organs in sheep. Third, there is the issue of breeding and animal availability during certain times in the year only. Even with hormonal treatment, results of sheep reproduction such as pregnancy rate and vitality of lambs (fetuses) are satisfactory only a few months around natural breeding seasons.[53] Hence, experimental scheduling requires careful planning with the years split into a fall and spring seasons.

A solution can be to establish September-to-November and an April-to-June experimental 'sheep seasons'. This issue is also a virtue, as it allows for time to analyze the many data collected during each experimental season.

There is a number of factors contributing to the significance with respect to existing methods. The morphometric, cardiovascular and blood gas data presented were within the range for the species [54,55] and resemble those of human species [56], a major advantage of this animal model. One exception is the higher rate of multiple pregnancies compared to human twinning. [57] This however is also a virtue of the model, as studying the effects of twinning on fetal development is an important biomedical task.[55,58,59] Very low levels of post-operative inflammation as measured by IL-6 and TNF-α ELISAs combined with recovery of acid-base status indicate that fetal surgical instrumentation is well tolerated and the post-operative recovery period of 72 hr is adequate to ensure a stable baseline condition of the fetal sheep prior to commencing an experiment. High percentage of spontaneous moderate chronic hypoxia in near-term fetal sheep renders them an interesting model for studying the chronic effects of the human antenatal hypoxia and inflammation on fetal and perinatal development, such as *e.g.,* IUGR, and perinatal insults, such as inflammation and acute asphyxia. [60,61,62] Several IUGR sheep models are used, some relying on spontaneous hypoxia, some inducing it by placental embolization, for example. [16,63-66] On the other hand, severe hypoxia prior to start of an experiment may also be an exclusion criterion in cases where *e.g.,* cardiovascular or central nervous systems are to be studied, as here the responses of chronically hypoxic fetuses are known to differ from those who are normoxic.[60] Another important application is the study of prenatal maternal stress impact on fetal and postnatal development. [5,67] Finally, as can be seen in the numerous cited publications with this model, the fetal instrumentation can be made throughout a wide range of gestational ages anywhere from ~70 to 135 dGA corresponding to mid-gestation – near-term studies of fetal development. With the advancing gestational age, instrumentation of ever increasing complexity are possible, but considerations of the duration of surgical instrumentation need to be weighed against the need to obtain a number of multivariate recordings from the same fetus.

A number of very promising future applications of the technique presented is derived from the ever-growing number of sheep-specific molecular biology reagents and recent sheep genome sequencing. These recent developments have further promoted this animal model to be a very promising and powerful approach to understanding healthy and pathological human fetal development on various scales of organization, from (epi)genome to integrative physiology. [68 69-74]

Disclosures

No disclosures have been made.

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