1% of the clinical dose used for antenatal steroid therapy is sufficient to induce lung maturation when administered directly to the preterm ovine fetus.
[DISCLOSURE STATEMENT]

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We used direct fetal administration of an ultra-low dose of betamethasone to demonstrate that a very low amplitude exposure to steroid treatment is sufficient for fetal lung maturation.

Direct fetal steroid administration for lung maturation.

Figure 7A/B (DFI vs MI levels)
[ABSTRACT]

Background: Antenatal steroids (ANS) are routinely administered to women judged to be at imminent risk of preterm delivery. Their principal benefit is precocious functional maturation of the preterm fetal lung. Current dosing regimens expose the mother and fetus to high steroid levels that may be unnecessary, increasing the potential risks of disruption to the maternal and fetal hypothalamic-pituitary-adrenal (HPA) axis and glucose regulation, alterations in placental function, and reduced fetal growth.

Objective: Using a sheep model of pregnancy, we tested the hypothesis that direct fetal administration of an ultra-low dose course of betamethasone phosphate (approximately 0.33 mg) would be sufficient to elicit functional maturation of the fetal lung.

Study design: A jugular catheter was installed in singleton ovine fetuses at 122d gestation under general anaesthesia. Animals were randomised to receive either: i) fetal intravenous betamethasone phosphate to target fetal plasma betamethasone mean levels of 2 ng/ml for 26 hours (Fetal Treatment Group; n=16); ii) fetal intravenous saline for 26 hours and two maternal intramuscular injections of 0.25 mg/kg betamethasone phosphate + betamethasone acetate, simulating a standard clinical treatment (Maternal Treatment Group; n=12); or iii) fetal intravenous saline only for 26 hours (Negative Control Group; n=10). Fetuses were delivered 48 hours after surgery, ventilated for 30 min to allow collection of lung function and physiological data, and euthanised. Quantitative PCR and western blots were used to assess markers of lung maturation.

Results: The average total betamethasone phosphate dose for the Fetal Treatment Group was 1 % (0.3 mg) of the Maternal Treatment Group (31 mg betamethasone phosphate + betamethasone acetate). At 30 minutes of ventilation, arterial pCO2, pH, heart rate and ventilation efficacy index
(VEI) were significantly (p<0.05) and equivalently improved in both the Fetal Treatment Group and Maternal Treatment Group, relative to the Negative Control Group. Similarly, SP-A, SP-C and AQ-S mRNA expression was significantly higher in both the Fetal Treatment Group and Maternal Treatment Group, relative to Negative Control.

**Conclusion:** Maternal steroid administration was not required to generate preterm fetal lung maturation in sheep. Using a low dose and targeting steroid treatments directly to the fetus has the potential to significantly reduce maternal exposures, whilst simultaneously reducing the potential risk of adverse outcomes associated with current clinical dosing regimens.

**[KEYWORDS]**

Antenatal steroids, lung maturation, preterm birth, fetus, sheep
[BACKGROUND]

Preterm birth (delivery before 37 completed weeks’ gestation) is a major cause of mortality and lasting morbidity. In high- and middle-income countries, being born preterm is the leading cause of under-five death(1). The immature preterm lung hinders adaptation to ex-uterine life, and as a result, breathing difficulties are a primary cause of death and lasting disease in preterm infants. (2-6).

Steroids are one of the most extensively used drugs in pregnancy. Antenatal steroid (ANS) treatment, which involves the use of single or repeat courses of synthetic (fluorinated) glucocorticoids, is standard care for women 24-34<sup>6</sup> weeks’ gestation at imminent risk of preterm delivery (7-9). Of late, there has been increased interest in ANS use in late preterm deliveries (34-38 weeks’ gestation), and in the setting of term deliveries by caesarean section(10, 11). Glucocorticoids exert pleiotropic effects, but predominantly act clinically to accelerate functional development of the immature lung, improving lung compliance and enhancing gas exchange by inducing the expression of surfactant proteins, saturated phosphatidylcholines, alveolar ion and water transport channels, thinning the mesenchyme and decreasing alveolar septation(12). ANS therapy (before 35 weeks’ gestation) has been found to reduce the risk of respiratory distress syndrome (RDS), intraventricular hemorrhage (IVH) and neonatal mortality(13).

The first ANS randomized control trial, published nearly fifty years ago, called for additional studies to gain further understanding of the ‘mode of action of glucocorticoids, on better selection of patients and on more effective control of uterine activity’ to allow more efficacious application of this therapy(14). Despite being in use for over 50 years, ANS treatment remains largely un-optimized. ANS therapy disturbs the materno-fetal HPA axis and glucose regulation, alters placental transport, and disrupts fetal growth and development (lower birth weights, smaller head circumferences) in a dose-dependent manner(15-17). Further research has linked fetal ANS exposure to potential long-
term adverse health outcomes including insulin resistance, obesity and cognitive and behavioral challenges (4, 5, 18-20).

Today a variety of different ANS dosing regimens are employed worldwide; dexamethasone phosphate (WHO recommended agent, use primarily in low and middle income countries (9)), the combined administration of betamethasone phosphate and betamethasone acetate (Australia, USA, much of Europe) or betamethasone phosphate alone (UK, Japan), used at varying doses and administration intervals (13). The one uniting feature of these regimens is that, based on recent data, they all employ what are increasingly viewed as unnecessarily high doses of steroid (21). A study by Kemp et al., using an ovine model of pregnancy, found that a constant low steroid exposure (maternal administration to achieve ~1-4 ng/mL fetal plasma betamethasone for at least 26h) was sufficient to generate fetal lung maturation, and that the additional high peak fetal steroid concentrations achieved with current clinical treatment regimens (i.e. 2 x 11.4 mg maternal treatments of betamethasone phosphate + acetate, spaced by 24 hours) does not additionally benefit the fetus (12). Consistent with earlier predictions by Jusko and colleagues (22), the study concluded that a maternally-administered steroid dose some 70 % less than current clinical treatments could generate an equivalent degree of lung maturation. Interest in reducing the dose of steroids used in ANS is reflected in the forthcoming ACTION-III trial, which will assess the efficacy of a low-dose betamethasone phosphate-based regimen (4 x 2 mg maternal intramuscular doses of betamethasone phosphate spaced by 12 hours) against the WHO-endorsed 4 x 6mg dexamethasone phosphate regimen (23). As a general principle the human fetus should be exposed to the lowest possible doses of any drug to achieve the desired effect.

Dose optimization is, along with judicious selection of recipient patients, likely key to improving the safety and efficacy of antenatal steroid therapy. Using a chronically catheterized sheep model of pregnancy this study aimed to demonstrate that: i) maternal treatment with ANS was not required
to generate fetal lung maturation; and ii) direct fetal administration of an ultra-low dose of betamethasone can elicit functional maturation of the preterm sheep fetal lung.

[METHODS]

Animal studies

All protocols were reviewed and approved by the animal ethics committee of The University of Western Australia (RA/3/100/1705). As previously(12), we sought to target and maintain a fetal plasma betamethasone concentration of 1-4 ng/mL. However, in the present study, we administered a steroid infusion directly to the fetus. The study consisted of two arms:

i) a pharmacokinetic (PK) study to generate the data necessary to model materno-fetal drug exposures deriving from a fetal intravenous administration of betamethasone, allowing determination of an appropriate dosing regimen and;

ii) A pharmacodynamic (PD) efficacy study of lung maturation to compare current clinical dosing to direct fetal administration of a low dose of corticosteroid.

Pregnant ewes were provided by a single supplier and experiments were performed during the normal breeding season. Ewes were date-mated and only ewes carrying a single fetus were used. At 115 ± 1 days’ gestation, all ewes received an intramuscular (IM) dose of 150 mg medroxyprogesterone acetate (Depo-Ralovera®; Pfizer, West Ryde, NSW, Australia). Previous studies show that medroxyprogesterone acetate can reduce the risk of preterm labor, but does not alter lung function in control animals or animals treated with betamethasone (24).

Animals were randomized to one of two study arms:

i) Pharmacokinetic Study: This study consisted of 6 ewes each carrying singleton pregnancies at 120 ± 1 days’ gestation. The ewes underwent aseptic recovery surgery to place a maternal jugular
catheter and fetal carotid and jugular catheters using previously described methods(25). The 
externalized catheters were secured in a container adhered to the back of the ewe. Animals 
recovered overnight following surgery. Each of the six fetuses was given a loading dose (intravenous 
bolus) of 60 µg betamethasone phosphate via the carotid artery catheter, followed by a constant 
infusion maintenance dose of 60 µg/hr for 13 hours. An elevated fetal concentration was selected to 
ensure that fetal betamethasone levels remained above the assay limit of detection to ensure 
modelling and efficacy dose calculations could be performed. Serial maternal and fetal (using the 
jugular catheter) blood samples were collected over a 30-hour period to measure drug levels 
(collection time points; 0, 1, 2, 4, 6, 8, 12, 13, 13.5, 14, 16, 24, 26, 28, 30 hours). Samples were 
promptly centrifuged at 3000 x g to separate plasma, which was then immediately frozen and stored 
at -80 °C for subsequent analysis. At 30 hours the ewe and fetus were euthanized. Betamethasone 
levels were determined using liquid chromatography mass spectrometry (LCMS) as reported 
previously(21).

ii) Efficacy Study: This study consisted of 38 ewes, each carrying a single fetus at 120 ± 1 days' 
gestation. The ewes underwent surgery to place a fetal jugular catheter as previously described 
(25). The externalized catheter was secured in a container adhered to the back of the ewe. Ewes 
were immediately randomized to one of three groups and all treatments were commenced 
immediately following the completion of surgery (Figure 1):

a) Fetal Treatment Group. Fetuses received an intravenous loading dose of betamethasone 
phosphate (Linosol® Injection, Wakamoto Pharmaceutical, Japan), followed by a continuous 
intravenous infusion of betamethasone phosphate to target fetal plasma betamethasone levels of 1-
4 ng/mL for 26 hours (n=16). The loading (18 µg) and maintenance infusion (12 µg/hr for 26 hours) 
doses were calculated from plasma concentration-time data generated in the initial Pharmacokinetic 
Study;
b) Maternal Treatment Group. Fetuses received an intravenous loading dose of saline, followed by a constant saline infusion for 26 hours. The volume and rate of saline administration was equivalent to that received by fetuses in the Fetal Treatment and Negative Control Groups. In addition, ewes were administered two intramuscular injections of 0.25 mg/kg betamethasone phosphate + betamethasone acetate (Celestone® Chronodose®, Merck Sharp & Dohme, Australia) spaced by 24 hours (n=12); or
e) Negative Control Group. Fetuses received an intravenous loading dose of saline, followed by a constant saline infusion for 26 hours. Volume and rate of saline administration was equivalent to that received by fetuses in the Fetal Treatment and Maternal Treatment Groups. (n=10).

Mean betamethasone dose was calculated for the Fetal Treatment Group and Maternal Treatment Group as follows, a) Fetal Treatment Group: final infusion volumes were recorded at 26 hour to determine total betamethasone dose delivered to each fetus and used to calculate mean dose for the group, b) Maternal Treatment Group: ewes were weighed prior to surgery to calculate a tailored dose of 0.25 mg/kg betamethasone phosphate + betamethasone acetate to be given at the completion of surgery, individual ewe dose was used to calculate mean dose for the group.

Fetuses were delivered under terminal anesthesia 48 hours after initiation of treatment protocol, ventilated for 30 min for measurement of lung function and physiological data, and euthanized as described previously.

Ventilation

At 122 ± 1 days' gestation, and precisely 48 hours post-surgery / initiation of treatment protocol, pregnant ewes received an intravenous injection of midazolam (0.5 mg/kg) and ketamine (10 mg/kg) followed by a 3 mL spinal injection of lidocaine (20 mg/mL). Lambs were then surgically delivered, administered an IM dose of ketamine (10 mg/kg) and intubated via tracheostomy using a 4.5 mm
endotracheal tube which was secured in place to create an airtight seal. Lambs were then dried and weighed, before being placed in a temperature controlled radiant warming bed (Cosy Cot, Fisher & Paykel Healthcare, New Zealand). All lambs were ventilated for 30 mins using Acutronic Fabian infant ventilators (Acutronic Medical System, Hirzel, Switzerland) delivering heated and humidified oxygen (100%). The initial ventilation parameters were as follows: peak inspiratory pressure (PIP) of 35 cmH₂O, positive end expiratory pressure (PEEP) of 5 cmH₂O, respiratory rate of 50 breaths per minute and an inspiratory time of 0.5 seconds; ventilation parameters are determined by data from previous studies and are kept uniform to allow for comparison between animals and future experiments. A maximum tidal volume of 7.8 mL/kg was targeted by adjusting peak inspiratory pressure. An umbilical artery catheter was placed to facilitate measurement of arterial blood for pH, pO₂, pCO₂, heart rate and blood pressure during ventilation. Ventilation data (compliance, tidal volume, PIP) was collected at 10-, 20-, and 30-minute time increments. Ventilation efficacy index (VEI) (an integrated measurement of ventilation) was calculated as follows: $VEI = 3800 / \text{[respiratory rate (PIP – PEEP) x pCO₂ (mmHg)]} $(26). The investigators who performed ventilations were blind to control or treatment group.

Necropsy

Ewes and lambs were euthanized with 160 mg/kg sodium pentobarbital. Lambs were weighed to record a post ventilation weight prior to necropsy. The lamb’s chest was opened to visualize the lungs, and static lung compliance was measured by inflation from 0 to 40 cm H₂O followed by controlled deflation to 0 cm. The lungs were then removed and weighed separately. The right lower lobe was dissected and frozen for molecular and protein studies.

Measurement of transcript expression changes in the fetal lung

Ribonucleic acid (RNA) was extracted from lung tissue (right lower lobe) using a RNeasy® Plus Mini Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer’s instructions.
concentration of extracted RNA was determined using a broad-range acid quantitation kit (Life
Technologies, Carlsbad, CA) and a Qubit 2.0 fluorometer (Life Technologies, Carlsbad, CA). RNA
extracts were diluted in nuclease-free water (Life Technologies) to achieve a final concentration of
25 ng/μL.

Quantitative polymerase chain reaction (qPCR) cycling was performed with ovine-specific TAQMAN
probe and primer sets (Applied Biosystems, Foster City, CA) using a Step One Real-Time PCR system
in accordance with manufacturer’s instructions. Messenger RNA transcripts for surfactant protein A
(SFTPA), surfactant protein B (SFTPA), surfactant protein C (SFTPC), surfactant protein D (SFTPD),
aquaporin 1 (AQP1), aquaporin 5 (AQP5), epithelial sodium channel subunits B (SCN1B) were
measured. Amplification data for each gene was normalized to ribosomal protein 18s RNA. Delta
quantification cycle values were used to determine relative expression of transcripts and for
statistical analyses of between-group differences. Data is presented graphically as fold change
compared to the negative control group.

Liquid Chromatography Mass Spectrometry

Extraction of plasma samples and betamethasone standards (200, 100, 40, 20, 10, 2, 1, 0 ng/ml)
were performed as follows: 50 μL of sample was added to 50 μL of internal standard (deuterated
betamethasone, 50 ng/ml in 50:50 methanol: water + 0.1% formic acid) as described previously(21).
Samples were vortexed for 10 seconds and incubated at room temperature (RT) for 5 min. 1 mL of
the solvent methyl tert-butyl ether (MTBE) was added, samples were sealed and vortexed for 2 min
before being centrifuged at 3000 rpm for 10 min. 700 μL of clear sample was transferred to glass
autosampler vials, dried under vacuum at 3000 rpm for 30 min at 37°C, before being reconstituted in
70 μL in 50:50 methanol: water + 0.1 % formic acid. Samples were capped and incubated with gentle
shaking for 10 min at 50 °C, then analyzed.
Protein Extraction and Analysis
Protein was extracted from fetal lung tissue and analyzed for surfactant proteins and glucocorticoid receptor-A according to methods previously optimized and published (27). Protein in tissue protein extraction reagent (T-PER) buffer was used for measurement of surfactant proteins (SFTPα, SFTPβ and SFTPc) and protein in radioimmunoprecipitation assay (RIPA) buffer was used for measurement of glucocorticoid receptor A (NR3C1). A XCell SureLock Mini-Cell Electrophoresis System (Life Sciences) was used for electrophoresis and membrane transfer. Membranes were analyzed using an iBright FL100 Imaging System (Invitrogen). Target band concentrations were measured and normalized by total protein concentration. A standard quality control sample was used across all membranes to normalize values.

In silico Pharmacokinetic Modelling
A pharmacokinetic model using a central, peripheral, and fetal compartment was developed to describe maternal and fetal concentrations following administration of betamethasone. Concentrations-time profiles to support the model were previously attained following intravenous infusion, intramuscular, and oral maternal administration of various forms of betamethasone (12, 21, 28, 29). Once the pharmacokinetic study completed the model was further updated to include fetal infusion and simulate the dose regimen to attain 2 ng/mL in the fetus.

Statistical Analysis
Statistical analysis was performed using IBM SPSS Statistics for Windows, version 25.0 (IBM Corp, Armonk, NY). Shapiro-Wilk test was used to test for normality of data before further analysis. A Kruskal Wallis test or one-way analysis of variance (ANOVA) were conducted according to normality (non-parametric or parametric data), followed by Tukey’s or Dunnett’s post-hoc tests. A t-test or
Mann-Whitney U test were used according to normality, to determine significant difference in hematological data (cortisol analysis) when values were only available for two groups. A chi-square test was used for comparing sex between groups.

[RESULTS]

i) PK study: We were unable to install one of the six maternal catheters. Samples were thus collected from five ewes and six fetuses.

Maternal and fetal plasma betamethasone levels were analyzed by LC-MS (Figure 2). The maximum fetal betamethasone concentration was 11.15 ng/mL, recorded at twelve hours. The maximum maternal concentration was 6.16 ng/mL recorded at thirteen hours. After the infusion ended, plasma betamethasone concentrations declined faster in the fetus compared to the ewe, suggesting active transplacental efflux. The plasma concentration-time data were used to design the dosing regimen for the efficacy study.

ii) Efficacy study: Thirty-eight out of forty lambs completed the protocol. One fetus was found to be of an incorrect gestational age (based on appearance and weight) and was removed from the study. An equipment error with an infusion pump resulted in a second fetus not completing its allocated treatment protocol, and it was also removed from subsequent analyses. As outlined above, animals were assigned to one of three groups (Figure 1). For the Fetal Treatment Group, an 18 μg betamethasone phosphate loading dose and a maintenance dose of 12 μg/hr of betamethasone phosphate administered for 26 hours was used to target a constant mean fetal plasma betamethasone concentration of 2.0 ng/mL (90% CI, 1.62-2.83).
Delivery data

All three groups were analyzed for differences in delivery metrics, with a p value of <0.05 considered significant (Table 1). There was no significant difference in fetal sex, gestational age, birthweight, or lung weight between the groups. There was a significant difference in cord blood neutrophils (mean 1.58 ± 0.52 vs 0.60 ± 0.22, p<0.001, 95% CI -1.5 - -0.45) and cord blood lymphocytes (mean 1.34 ± 0.73 vs 2.44 ± 0.68 , p=0.003, 95% CI 0.34-1.85) between the Maternal Treatment Group and Negative Control Group.

Hematological analysis of maternal and fetal blood

Maternal and cord blood samples were collected at delivery to measure plasma cortisol and adrenocorticotropic hormone (ACTH) levels (Table 2). Cortisol levels in the Maternal Treatment Group were below the limit of detection (<5.5 nmol/L) in all the fetal and maternal blood samples. Cortisol levels were below the limit of detection in four of the sixteen fetal blood samples from the Fetal Treatment Group and one of the ten fetal blood samples in the Negative Control Group; these animals were assigned an arbitrary cortisol level of 5.5 nmol/L for the purpose of subsequent analyses.

Analysis showed no significant difference in plasma cortisol levels between the fetal treatment group and negative control group (Table 2). ACTH levels were below the limit of detection (<5.5 nmol/L) in one of twelve fetal blood samples and two of twelve maternal blood samples in the Maternal Treatment Group, these animals were assigned an arbitrary ACTH level of 5.5 nmol/L for the purpose of analysis. There was a statistically significant difference in fetal plasma ACTH levels between the Fetal Treatment Group and Negative Control Group animals (median difference 371, p<0.001), however no significant difference was detected in maternal ACTH levels between these two groups. Maternal treatment was found to significantly suppress ACTH levels in both the ewe (mean 12.42 ± 6.92 vs 231.10 ± 132.00 , p=0.002, 95% CI -357.65 - -79.55) and fetus (median 6.50,
IQR 3.75 vs 413.50, IQR 602.75, p<0.001) when compared to the Negative Control Group (Table 2).

The Fetal Treatment did not change maternal ACTH, therefore the cortisol concentration was below the pharmacodynamic relevant value.

Betamethasone measurement

Fetal and maternal plasma betamethasone levels at delivery were measured using LC-MS. The limit of detection for our assay was defined as a betamethasone plasma concentration of 1ng/ml with a signal to noise ratio of >10:1. Fetal and maternal betamethasone plasma levels at delivery were below the limit of detection in the Fetal Treatment Group. Animals in the Maternal Treatment Group had measurable levels of betamethasone at delivery in both the fetal and maternal compartments (Table 2). No betamethasone was measurable in the maternal or fetal circulation in Negative Control Group animals.

Ventilation Data (30 minutes)

There was a significant difference in pH (mean 6.96 ± 0.20 vs 6.79 ± 0.09, p=0.033, 95% CI -0.33 - 0.01), PaCO₂ (mean 89.67 ± 41.80 vs 140.16 ± 21.07, p=0.003, 95% CI 15.61-85.37) and heart rate (mean 132.69 ± 30.30 vs 177.90 ± 15.80, p=0.002, 95% CI 15.65-74.77) between the Fetal Treatment Group and Negative Control Group. There was a significant difference in pH (mean 7.09 ± 0.15 vs 6.79 ± 0.09, p<0.001, 95% CI -0.48 - -0.14), PaCO₂ (mean 74.82 ± 36.69 vs 140.16 ± 21.07, p<0.001, 95% CI 30.68-104.77) and heart rate (mean 142.2 ± 37.64 vs 177.9 ± 15.80, p=0.038, 95% CI 1.50 - 64.30) between the Maternal Treatment Group and the Negative Control Group. There was no significant difference in PaO₂ between groups (Figure3).

Animals in the Fetal Treatment Group and the Maternal Treatment Group were classified as being either treatment responders- or treatment non-responders on the basis of their 30-minute PaCO₂ measurements. To make this determination, we decided a priori to use a threshold 30-minute PaCO₂
value of two standard deviations below that of the mean value of normally distributed Negative Control Group animals as previously described (29, 30). On this basis, steroid-treated animals that had a 30-minute PaCO₂ value within two standard deviations below the mean Negative Control Group PaCO₂ 30-minute value were considered non-responders. Steroid-treated animals with more extreme values (i.e. >2 standard deviations below the mean Negative Control Group 30-minute PaCO₂ value) were considered responders. Based on Negative Control Group data, the response cut-off value at 30-minutes of ventilation was PaCO₂< 100. Based on this, 73% (n=11/16) of the Fetal Treatment Group were classified as responders, compared to 83% (n=10/12) of the Maternal Treatment Group. No animals from the Negative Control Group were classified as responders.

There was a significant difference in VEI between the Fetal Treatment Group and the Negative Control Group (median 0.03, IQR 0.03 vs 0.02, IQR <0.01, p=0.37). There was a significant different between the Maternal Treatment Group and Negative Control Group for Compliance (mean 0.46 ± 0.16 vs 0.27 ± 0.08, p=0.029, 95% CI -0.37--0.02), Vt (mean 14.03 ± 4.48 vs 8.37 ± 2.39, p=0.019, 95% CI -10.48--0.83), VEI (mean 0.04 ± 0.02 vs 0.02 ± 0.003, p=0.007, 95% CI -0.04--0.006) and V40 (mean 12.25 ± 2.85 vs 7.24 ± 1.82, p<0.001, 95% CI -7.73--2.29) (Figure 4).

Quantitative polymerase chain reaction (qPCR) analysis of transcript expression changes in the fetal lung

There was a significant difference in the expression of SFTPA (ΔCT mean 14.16 ± 0.62 vs 15.14 ± 0.35, p=0.006, 95% CI 0.25-1.71) and SFTPC (ΔCT mean 9.35 ± 0.39 vs 10.07 ± 0.59 difference 0.73, p=0.001, 95% CI 0.29-1.16) between the Fetal Treatment Group and the Negative Control Group animals. There was a significant difference between the Maternal Treatment Group and the Negative Control Group for SFTPA (ΔCT mean 13.21 ± 0.50 vs 15.14 ± 0.35 , p<0.001, 95% CI 1.16-2.7), SFTPB (ΔCT mean 12.74 ± 0.19 vs 13.98 ± 0.53, p<0.001, 95% CI 0.81-1.67), SFTPC (ΔCT median 8.73, IQR 0.41 vs 9.93, IQR 0.71, p<0.001) and SFTPĐ (ΔCT mean 14.04 ± 0.61 vs 14.90 ± 0.56, p=0.19,
95% CI 0.13-1.161). There was a significant difference in the expression of AQP5 between the Fetal Treatment Group and the Negative Control Group (ΔCT mean 15.99 ± 0.27 vs 16.40 ± 0.29, p=0.014, 95% CI 0.07-0.74), There was a significant difference in relative expression of AQP1 (ΔCT mean 15.80 ± 0.29 vs 16.55 ± 0.37, p<0.001, 95% CI 0.37-1.13), AQP5 (ΔCT mean 15.68 ± 0.44 vs 16.40 ± 0.29, p<0.001, 95% CI 0.36-1.07), and SCNN1B (ΔCT mean 15.32 ± 0.46 vs 16.55 ± 0.54, p<0.001, 95% CI 0.38-1.79), between the Maternal Treatment Group and the Negative Control Group (Figure 5).

**Western Blot analysis of lung tissue**

NR3C1, SFTPα, and SFTPβ bands were confirmed at 90 kDa, 35 kDa, and 19 kDa, respectively. Band volumes were determined to be proportional to total protein concentration. There was a significant difference in SFTPβ protein concentration between the Fetal Treatment and Negative Control Groups (mean 0.88 ± 0.28 vs 0.52 ± 0.09, p=0.012, 95% CI 0.07-0.66). There was a significant different in NR3C1 (mean 0.76 ± 0.27 vs 1.14 ± 0.36, p=0.05, 95% CI -0.76- -0.0002), SFTPα (mean 1.07 ± 0.39 vs 0.66 ± 0.18, p=0.005, 95% CI 0.12-0.72) and SFTPβ (mean 1.19 ± 0.41 vs 0.52 ± 0.09, p<0.001, 95% CI 0.36-0.98) between the Maternal Treatment Group and the Negative Control Group animals.

**In silico Modelling**

Pharmacokinetic modeling for the fetal treatment group (using a constant betamethasone infusion into fetal circulation), targeting a fetal plasma betamethasone concentration of 1-4 ng/ml for 26 hours, was compared to modelling for a maternal infusion treatment (using a constant betamethasone infusion into maternal circulation) by Kemp et al. targeting the same fetal plasma betamethasone concentration(12). For the Fetal Treatment Group using a direct fetal infusion (DFI), fetal plasma betamethasone concentration was predicted to drop below 0.1 ng/ml at 32 hours after initiation of infusion. For the animals in the previously published maternal infusion (MI) study, fetal
plasma betamethasone concentration was predicted to remain elevated above 0.1 ng/mL beyond 57
hours (Figure 7A, 7B).

[COMMENTS]

Principal Findings

The principal findings of this study are:

i) that maternal steroid administration is not required to generate fetal lung maturation. Using a
direct fetal dosing approach, we were able to generate lung maturation, significantly (p<0.05) better
than that observed in the Negative Control Group. Using the previously established PaCO₂ cutoff to
determine treatment responders vs. non-responders, 73% of lambs in the Fetal Treatment Group
responded to the treatment;

ii) that a fetal betamethasone phosphate exposure equivalent to 1% of the current maternal clinical
dosing (betamethasone phosphate + betamethasone acetate) is sufficient to drive fetal lung
maturation; and

iii) that lung maturation induced by maternal administration is more robust than that achieved with
direct fetal administration.

Based on our in silico modelling (Figure 7), we speculate that the observed difference in treatment
performance between the Fetal and Maternal Treatment Groups is due to an insufficient duration of
 supra-threshold fetal betamethasone exposure in the Fetal Treatment Group. It is tempting to
speculate that the lower limit of a continuous fetal steroid exposure (ng/mL betamethasone in fetal
plasma) is significantly lower than that originally predicted, and may be as low as 0.1ng/mL.

Results and research implications
The average betamethasone dose for the Fetal Treatment Group was 0.3 mg administered over 26 hours, compared to 31 mg in the Maternal Treatment Group (2 x IM 0.25 mg/kg betamethasone phosphate and acetate) administered over 24 hours. Direct fetal steroid dosing did promote lung maturation; however, lung maturation was not equivalent in either magnitude or reliability to the Maternal Treatment Group. We have previously shown that a fetal betamethasone exposure of ~2 ng/mL, generated from a constant maternal infusion of betamethasone phosphate for 26 hours, promoted lung maturation equivalent to the current clinical dosing regimen (12). We undertook in silico modelling of the drug exposures from our earlier study, and of those (direct fetal exposures) presented in this report. As shown in Figure 7, in the setting of a maternal infusion, fetal steroid exposure was predicted to remain elevated above 0.1 ng/ml until delivery at 48 hours post-treatment initiation, despite cessation of the maternal infusion at 26 hours (Figure 7B). Maternal betamethasone levels were elevated above 1ng/ml past 48 hours, consistent with the maternal circulation acting as a reservoir delivering steroid to the fetus long after the infusion ended. In contrast, exposures in the current study saw fetal betamethasone concentration fall below 0.1 ng/mL by 32 hours and maternal betamethasone concentrations, which never exceeded 1ng/ml, dropping below 0.5ng/ml at 30 hours. These data could indicate that a longer, low-amplitude exposure time is required to generate fetal lung maturation equivalent to that of current clinical treatment.

This study not only provides further evidence to show that maternal administration to ANS is unnecessary, that the data suggest that the efficacious floor for fetal steroid exposures in ANS therapy is likely lower than originally anticipated. Determining the minimum efficacious dose required to generate fetal lung maturation, while reducing the risks of fetal growth restrictions and other maternal-fetal complications, is not only useful for optimizing current treatment regimens, but could form the basis of a non-invasive fetal targeted therapy to reduce maternal and fetal exposure to unnecessarily high levels of steroids. Although there is no published record of direct fetal ANS...
treatment in large human cohorts, previous studies using a small sample of human fetuses or animal
models have trialed direct fetal therapy.

In 1969 Liggins investigated parturition in the sheep in response to different glucocorticoid
regimens, including dexamethasone, deoxycorticosterone, corticosterone which were infused
directly into the fetal circulation(31). Liggins found that lambs born preterm following an infusion of
steroids survived longer than lambs not exposure to steroids in utero and those born between 117-
123 days (equivalent to ~32 weeks human gestation) 1-7 days after receiving dexamethasone had
partial lung aeration; thought to be the result of increased surfactant activity.

A study by Jobe et al. in 1993 assessed fetal lung responses to ultrasound guided corticosteroid
treatment into the leg of a premature fetal sheep, followed by delivery at a gestational age of 128
days(32). Lambs treated with a single dose of betamethasone 48 hours before delivery had
significantly better lung compliance and improved VEI compared to those that received saline. A
follow-on study comparing direct fetal administration vs. maternally administered ANS found that
lambs born to ewes who received direct fetal ANS did not experience the same growth restriction
and overall lower birth weight and weight of major organs seen with maternally delivered ANS. A
final pharmacokinetic study by Moss et al. found that direct fetal ANS injections resulted in high
peak fetal betamethasone concentrations that cleared quickly, however the same dose delivered
maternally resulted in a prolonged exposure which was sufficient to cause growth restriction in the
fetus(33). Two previous studies have employed the same techniques in humans, however sample
sizes were small and results were varied suggesting more work is required before a RCT is
feasible(34, 35).

Clinical implications

15 million babies are born preterm every year, with complications resulting from preterm birth a
major cause of mortality and lasting morbidity worldwide. Antenatal steroids are among the most
important and widely used drugs in pregnancy today, with dexamethasone listed on the WHO essential list of medicines for treatment of mothers at imminent risk of preterm delivery(9). More recent studies have raised increasing concerns about the potential harms caused by ANS therapy and the use of unnecessarily high levels of steroids, that seem to convey no further benefit to the mother or developing fetus(4, 13, 19, 36).

The use of repeat courses of ANS have been found to further exacerbate delivery effects, suggesting a possible dose dependent response between steroid dose, more so repeat dosing, and adverse health outcomes associated with ANS use. A trial reported by Crowther et al. showed a decrease in Z scores for weight (p=0.04) and head circumference (p=0.03) for babies who received multiple courses of ANS(37). Additionally, a RCT by Murphy (MACS trial) comparing single and repeat courses (every 14 days) of ANS found that infants exposed to multiple courses of steroids were significantly smaller at birth, weighing an average of 113 g less, with smaller head circumference and overall length(38). A 5 year follow up (MACS-5 trial) found that multiple courses of ANS was associated with greater risk of neurosensory disability among children subsequently born at term(37, 39).

ANS exposure has been found to cause adverse effects continuing into childhood including insulin resistance, obesity, and cognitive and behavioral issues (4, 5, 20, 40). Gyamfi-Bannerman et al. reported the clinical use of ANS for late preterm pregnancy was associated with increased risk of neonatal hypoglycemia(10). Neonatal hypoglycemia has been linked to increased risk of adverse long-term outcomes, including poor cognitive and motor function in childhood(18, 19). An association study by Raikkonen et al. found that children exposed to ANS and delivered at term were almost twice as likely to have a mental or behavioral disorder than children not exposed to ANS (12.01% vs 6.45%, p<0.001); this figure remained significant when only including children born preterm (14.59% vs 10.71% p<0.001)(40). The follow-up of the ASTECS trial assessing children once they reached school age (8-15 years of age), found that children whose mothers were treated with ANS before elective delivery at term were twice as likely to be in the lower quartile of academic
ability than those not exposed to steroids (17.7% vs 8.5%)(4). A study by Alexander et al. found exposure to ANS before delivery at term was associated with increased cortisol secretion to psychosocial stress in children aged 6-11 years(41).

Additionally, a 30 year follow up of the inaugural RCT by Liggins in 1972 found that antenatal betamethasone exposure resulted in adult insulin resistance, potentially increasing risk of developing diabetes and cardiovascular disease later in life(5, 20). The impact of these findings is, however, unclear and a socio-economic assessment of trial participants from the original New Zealand cohort failed to determine any material differences between steroid-treated and naïve adults(20).

A therapeutic regime that conveys benefit at a lower dose and peak materno-fetal steroid exposure would likely convey improved health outcomes. Our data show that a substantially reduced dose of steroids is associated with reduced disruption to the materno-fetal HPA axis and may reduce possible dose-dependent adverse health impacts to mother and fetus.

Strengths and limitations

It is important to note that the data presented herein were generated using a sheep model of pregnancy, using a small number (relative to a clinical efficacy study) of surgically manipulated animals. The direct translatability of our data to a human clinical model remains to be established, however a sheep model of pregnancy has been used extensively in the development of antenatal steroid therapy. This study was designed to act as a proof-of-concept study to inform a further dose optimization study on sheep.
Sample sizes were only powered to determine significant difference between treatment and non-treatment control groups. A large sample number would be required to determine significant differences or non-inferiority between the two treatment groups.

Conclusion

Strategies to reduce materno-fetal steroid exposures to an absolute minimum are likely to better balance desired improvements in fetal lung maturation with a minimum of disruption to fetal development, materno-fetal homeostasis, and to placental functions critical to normal fetal development, resulting in an improved overall treatment efficacy. Further research is required to find the lower limit of fetal steroid exposure necessary to achieve robust, durable lung maturation.

Carefully calibrated reductions in maternal and fetal steroid exposures have the potential to reduce adverse outcomes. These data will form the basis to develop a non-invasive antenatal steroid therapy employing a minimally efficacious dose of antenatal steroids.

[ACKNOWLEDGEMENT]

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**[TABLES]**

**Table 1: Control and Treatment Groups delivery data.**

An asterisk (*) indicates a significant difference (p<0.05) between a treatment group and the Negative Control Group.

**Table 2: Plasma cortisol, ACTH and plasma levels at delivery.**

An asterisk (*) indicates a significant difference (p<0.05) between a treatment group and the Negative Control Group.
[FIGURES]

Figure 1: Efficacy study treatment groups and dosing regimens.
Animals were divided into 3 treatment groups: i) Fetal Treatment group; ii) Maternal Treatment Group; or iii) Negative Control Group.

Figure 2: Maternal and fetal plasma betamethasone levels over 30 hours.
Mean plasma concentration time profiles in the Pharmacokinetic study following an infusion directly to the fetus with a loading dose of 60 µg and maintenance dose of 60 µg/hr for 13 hours. Blood samples were taken serially from maternal and fetal catheters up to 30 hours after the start of treatment.

Figure 3: Fetal arterial blood gas measurements and physiological data.
Arterial blood gas measurement and physiological parameters were recorded at 30 minutes of preterm lamb ventilation. A, PaO₂ (Fetal Treatment Group vs. Negative Control Group, mean difference -59.56 [95% CI, -135.66- 16.53]); Maternal Treatment vs. Negative Control Group, mean difference -75.77 [95% CI, -156.59-5.06]). B, PaCO₂ (Fetal Treatment Group vs. Negative Control Group, mean difference 50.49 [95% CI, -85.37- -15.61]); Maternal Treatment vs. Negative Control Group
800 Group, mean difference 67.73 [95% CI, 30.68-104.77]). C, pH (Fetal Treatment Group vs. Negative
801 Control Group, mean difference -0.17 [95% CI, -0.33- -0.01]; Maternal Treatment Group vs. Negative
802 Control Group, mean difference -0.31 [95% CI, -0.48- -0.14]). D, Heart rate (Fetal Treatment Group
803 vs. Negative Control Group, mean difference 45.22 [95% CI, 15.65-74.77]; Maternal Treatment
804 Group vs. Negative Control Group, mean difference 32.90 [95% CI, 1.50-64.29]). An asterisk (*)
805 indicates a significant difference (p<0.05) between the treatment group and the Negative Control
806 Group.
807
808 **Figure 4: Key physiological parameters after 30 minutes of ventilation**
809 Ventilation outcomes after 30 minutes of preterm lamb ventilation. A, Compliance (Fetal Treatment
810 Group vs. Negative Control Group, mean difference -0.15 [95% CI, -0.32-0.01]; Maternal Treatment
811 Group vs. Negative Control Group, mean difference -0.19 [95% CI, -0.37- -0.02]). B, Vt (Fetal
812 Treatment Group vs. Negative Control Group, mean difference -4.23 [95% CI, -8.77-0.31]; Maternal
813 Treatment Group vs. Negative Control Group, mean difference -5.66 [95% CI, -10.48- -0.83]). C, VEI
814 (Fetal Treatment Group vs. Negative Control Group, mean difference -0.02 [95% CI, -0.04- -0.001];
815 Maternal Treatment Group vs. Negative Control Group, mean difference -0.02 [95% CI, -0.04- -0.006]). D, V40 (Fetal Treatment Group vs. Negative Control Group, mean difference -5.12 [95% CI, -10.04- -0.21]; Maternal Treatment Group vs. Negative Control Group, mean difference -5.01 [95%
816 Cl, -10.34-0.32]). An asterisk (*) indicates a significant difference between the treatment group and
817 the Negative Control Group. An asterisk (*) indicates a significant difference (p<0.05) between the
818 treatment group and the Negative Control Group.
819
820 **Figure 5: Messenger RNA quantification in fold change relative to the Negative Control Group.**
Relative expression of mRNA transcript for surfactant proteins A, B, C and D and AQP1, AQP5 and SCNN1B. An asterisk (*) indicates a significant difference (p<0.05) between the treatment group and the Negative Control Group.

SFTP A, surfactant protein A; SFTP B, surfactant protein B; SFTP C, surfactant protein C; SFTP D, surfactant protein D. AQP1, aquaporin 1; AQP5 aquaporin 5; SCNN1B, sodium channel subunit B.

Figure 6: Total protein concentration in fold change relative to the Negative Control Group.

Western Blot analysis for SFTP A, SFTP C and NC3R1. An asterisk (*) indicates a significant difference between the treatment group and the Negative Control Group.

SFTP A, surfactant protein A; SFTP C, surfactant protein C; NC3R1, glucocorticoid receptor A.

Figure 7: Comparison of pharmacokinetic data for standard clinical treatment (2 x IM), direct fetal infusion (DFI) and previous maternal infusion (MI) studies.

A) The plasma betamethasone concentrations generated by the standard clinical treatment (2x IM) (2 x maternal IM of 0.25 mg/kg betamethasone phosphate + betamethasone acetate) is indicated by the light green (fetus) and dark green (ewe) dashed lines. The plasma betamethasone concentrations generated by the direct fetal infusion (DFI) (betamethasone phosphate loading dose of 18 µg + infusion of 12 µg/hr for 26 hours) is indicated by the light blue (fetus) and dark blue (ewe) dashed lines. The plasma betamethasone concentrations generated by the maternal infusion (MI) (betamethasone phosphate loading dose of 0.028mg/kg + infusion of 0.104mg/kg for 26 hours) are indicated by the light red (fetus) and dark red (ewe) dashed lines.

B) The purple box shows the current target fetal plasma betamethasone concentration for DFI and MI (1-4 ng /mL). The green box indicates the potential lowest dose for optimizing ANS therapy.
<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td>Control and Treatment Group Delivery Data</td>
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<tr>
<td>Treatment Group</td>
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<tr>
<td>Control Group</td>
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<tr>
<td>Gestation age (days)</td>
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<tr>
<td>Birthweight (kg)</td>
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<td>Sex (male/female)</td>
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<td>Cord pH</td>
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<td>Cord blood pCO2 (mm Hg)</td>
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<td>Lung weight (g/kg)</td>
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<tr>
<th>Cord Blood Values</th>
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<tr>
<td>Total WBC (x10⁹/L)</td>
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<tr>
<td>Neutrophils (x10⁹/L)</td>
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<td>Lymphocytes (x10⁹/L)</td>
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Values are expressed as mean ± standard deviation

*P < .05 vs control.
<table>
<thead>
<tr>
<th>TABLE 2</th>
<th>Cortisol and ACTH Data at Delivery</th>
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<tr>
<td></td>
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<tr>
<td>Control</td>
<td>Treatment</td>
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<td>Group</td>
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<tr>
<td><strong>Cortisol</strong></td>
<td></td>
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<tr>
<td>Fetal (cord blood)</td>
<td>14.3 ± 4.2</td>
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<tr>
<td>Maternal</td>
<td>44.5 ± 29</td>
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<tr>
<td><strong>ACTH</strong></td>
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<tr>
<td>Fetal (cord blood)</td>
<td>406.9 ± 313.9</td>
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<tr>
<td>Maternal</td>
<td>231.1 ± 132.0</td>
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<tr>
<td><strong>Plasma Steroid Levels at Delivery (ng/ml)</strong></td>
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<tr>
<td>Fetal (cord blood)</td>
<td>0</td>
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<tr>
<td>Maternal</td>
<td>0</td>
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</tbody>
</table>
Group

**Fetal Treatment** (Betamethasone Phosphate)
- Beta Phos Loading dose + Beta Phos infusion (to target 2ng/ml beta in fetal plasma).
  - T=-48hr

**Maternal Treatment** (Celestone)
- Celestone IM 0.25mg/kg + Saline Loading Dose + Saline Infusion
  - T=-48hr
- Celestone IM 0.25mg/kg
  - T=-24hr

**Negative Control** (Saline only)
- Saline Loading Dose + Saline infusion
  - T=-48hr
  - T=-24hr

Infusion End -22hr

Delivery 0hr
A

Compliance 30min (mL/cm H2O)

B

VT 30min (mL/kg)

C

VBT 30min

D

Volume at 40 cm H2O (mL/kg)

△ Negative Control ★ Maternal Treatment ▲ Fetal Treatment
Maternal Infusion (MI) and Direct Fetal Infusion (DFI) vs. Standard Clinical Treatment (2x IM)

- DFI- Fetal Plasma
- DFI- Maternal Plasma
- MI- Fetal Plasma
- MI- Maternal Plasma
- 2x IM- Fetal Plasma
- 2x IM-Maternal Plasma

Concentration (ng/ml) vs. Time (hours)
Maternal Infusion (MI) vs Direct Fetal Infusion (DFI)
(Target 2ng/ml Fetal Plasma Betamethasone Concentration)

B

INFINITY (28HRT)

1-4ng/ml

0.1-1ng/ml

MI- Maternal Plasma

MI- Fetal Plasma

DFI- Maternal Plasma

DFI- Fetal Plasma

Concentration (ng/ml)

Time (hours)