Variability in the Efficacy of a Standardized Antenatal Steroid Treatment is Not Due to Maternal or Fetal Plasma Drug Levels. Evidence from a Sheep Model of Pregnancy.

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[TITLE]

Variability in the Efficacy of a Standardized Antenatal Steroid Treatment is Not Due to Maternal or Fetal Plasma Drug Levels. Evidence from a Sheep Model of Pregnancy.

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[CONDENSATION]

In sheep and humans there is variability in the maturation of the preterm fetal lung in response to antenatal steroid treatment. In the present study this variation was not associated with differences in maternal or fetal steroid plasma concentrations.

[SHORT TITLE]

Variability in fetal sheep lung responses to antenatal steroids.

[AJOG at a Glance]

A. *Why was this study conducted?*

Antenatal steroids (ANS) are a widely used treatment to improve outcomes for preterm infants, principally by maturing the fetal lung and stabilizing the cardiovascular system. Treatment efficacy is highly variable, even when potentially confounding factors (i.e. treatment to delivery interval, agent, dose) are standardized. Hypothesizing that differences in materno-fetal steroid exposure contribute to observed variability in ANS treatment efficacy, we used a chronically catheterized sheep model of pregnancy and post-natal ventilation to test if the relationship between materno-fetal steroid exposure and functional lung maturation explained variation in lung maturation effects.

B. *What are the key findings?*

Following a main-group analysis to confirm overall effect, preterm lambs which had been exposed to a single maternal antenatal injection of 0.25 mg/kg betamethasone (as 1:1 betamethasone phosphate and betamethasone acetate) were stratified to either a responder

group or a non-responder group, based upon arterial $PaCO_2$ levels at 30 minutes ventilation being within two standard deviations of the mean value for Control Group (Saline only) fetuses. No differences in betamethasone distribution, clearance, or protein binding ratio were observed between responder and non-responder ANS treatment groups.

C. *What does this study add to what is already known?*

This study correlated individual maternal and fetal ANS drug exposures with treatment efficacy as determined by functional lung maturation. Our data showed that observed differences in ANS treatment efficacy between identically-dosed animals were not due to differences in materno-fetal drug exposure. Differences may be due to fetus or pregnancy-specific factors that modify individual steroid responses.

[SELECTED FIGURE]

Figure 5.

[ABSTRACT]

***Background*:** Antenatal steroids (ANS) are standard of care for women judged to be at imminent risk of preterm delivery. Worldwide, there is significant variation in ANS dosing strategy, selection for treatment criteria, and agent choice. This, combined with very limited optimization of ANS use *per se* means that treatment efficacy is highly variable and the rate of respiratory distress syndrome is decreased perhaps as little as 40%. In some cases, ANS use is associated with limited benefit and potential harm.

***Objective*:** We hypothesized that individual differences in maternal and fetal steroid exposure would contribute to observed variability in ANS treatment efficacy. Using a chronically catheterized sheep model of pregnancy, we aimed to explore the relationship between materno-fetal steroid exposure and ANS treatment efficacy as determined by functional lung maturation in preterm lambs undergoing ventilation.

***Methods*:** Ewes carrying a single fetus had surgery to catheterize a fetal and maternal jugular vein at 119 days' gestation. Animals recovered for 24h before being randomized to either: **i)** a single maternal intramuscular injection (IM) of 2ml saline (Negative Control Group, n=10); or **ii)** a single maternal IM of 0.25mg/kg betamethasone phosphate + acetate (ANS Group, n=20). Serial maternal and fetal plasma samples were collected from each animal over 48h before fetuses were delivered and ventilated for 30 minutes. Total and free plasma betamethasone concentration was measured by mass spectrometry. Fetal lung tissue was collected for analysis using quantitative polymerase chain reaction.

Results: One animal of the Control Group and one animal from the ANS Group had did not complete their treatment protocol and were removed from analyses. Animals in the ANS Group were divided into a Responder (n=12/19) Sub-Group and a Non-Responder

Sub-Group (n=7/19) using a cut-off of a $PaCO_2$ at 30 minutes ventilation within 2SD of the mean value from saline-treated Negative Control Group animals. While ANS improved fetal lung maturation in the undivided ANS group, and in the Responder Sub-Group both physiologically (blood gas and ventilation related data) and biochemically (mRNA expression related to fetal lung maturation), these values were not improved relative to saline-treated Control Group animals in the ANS Non-Responder Sub-Group. Interestingly, no differences in betamethasone distribution, clearance, or protein binding were identified between the ANS Responder and Non-Responder Sub-Groups.

***Conclusion*:** This study correlated individual materno-fetal steroid exposures with preterm lung maturation as determined by pulmonary ventilation. Herein, approximately 40% of preterm lambs exposed to antenatal steroids had lung maturation not significantly different to saline-treated Control Group animals. These non-responsive animals received maternal and fetal betamethasone exposures identical to animals that had a significant improvement in functional lung maturation. These data suggest that the efficacy of ANS therapy is not solely determined by materno-fetal drug levels, and that individual fetal or maternal factors may play a role in determining treatment outcomes in response to glucocorticoid-driven signaling.

[KEYWORDS]

Betamethasone, fetus, glucocorticoid, lamb, lung maturation, pharmacokinetics, preterm birth, protein binding, sheep.

[Text]

[BACKGROUND]

Preterm birth is a leading cause of neonatal morbidity and mortality. Antenatal steroids (ANS) are a standard of care for women at risk of delivering preterm. The American College of Obstetricians and Gynecologists, for example, recommends the use of '*a single course of corticosteroids for pregnant women between 24 0/7 weeks and 33 6/7 weeks of gestation who are at risk of preterm delivery within 7 days*'. [1] However despite extensive use, ANS dosing, choice of agent, and patient selection remains relatively unchanged and poorly unoptimized after almost 50 years of clinical use. [2] ANS treatment is considered among the most important therapies to improve outcomes for preterm babies and has been used widely since Liggins and Howie reported the beneficial effect of ANS in a cohort of babies born between 28 and 32 weeks' gestation in their landmark 1972 paper. [3] There is significant geographical variation in the usage of ANS; highlighting the different doses and agents employed (and derivative differences in materno-fetal pharmacokinetics and pharmacodynamics), the United Kingdom and Japan commonly use 12 mg maternal intramuscular injections of betamethasone phosphate given 24 hours apart. [4] The WHO-recommended dosing regimen is four 6 mg maternal intramuscular injections of dexamethasone phosphate given 12 hours apart for pregnant women who are at risk of preterm labor between 24 and 34 weeks of gestation. [5] In the United States, Australia, and much of Europe, a course of two maternal intramuscular injections of 12mg 1:1 betamethasone acetate and betamethasone phosphate spaced at 24 hours is commonly used. [1, 6]

A recent Cochrane systemic review reported that a single course of ANS treatment for singleton pregnancies between 26 and 34 weeks of gestation contributed to a significant reduction in adverse outcomes including perinatal and neonatal death, respiratory distress syndrome (RDS) and intraventricular hemorrhage.[6] When given to the right women and the right time, the benefit derived from ANS use are well-documented. However, efficacy is variable and there is evidence from both clinical and animal studies to suggest that unwanted side effects such as growth restriction and programming changes are associated with repeated ANS exposure. [7, 8] Current clinical dosing (12mg 1:1 betamethasone acetate and phosphate) achieves betamethasone concentrations in the fetal plasma peak at around 20 ng/ml in 2 hours after the first administration.[9] Using a sheep model of pregnancy, we have previously demonstrated that high dose ANS treatment is not necessary for lung maturation, and that maintaining low fetal plasma betamethasone concentration in the range of 1-4 ng/mL for a period of 26 or 36 hours resulted in fetal lung maturation equivalent to effects seen with the current clinical dose when delivery occurred 48h after treatment initiation.[10] Additionally, a 24 hour fetal ANS exposure (i.e. single clinical dose of betamethasone) was sufficient for lung maturation of 24-48 hour post-treatment deliverlies.[11]

The highly variable efficacy of ANS treatment is well documented clinically. [5, 6] We have also observed significant variation in our experimental animals, despite them being gestational age matched, receiving an identical weight-calibrated ANS dose, and being delivered for ventilation at an identical treatment to delivery interval. We hypothesized that these observed differences were due to individual differences in the distribution or clearance of steroids from the materno-fetal compartments. To test this hypothesis we used a

chronically catheterized sheep model of pregnancy to explore the relationship between materno-fetal steroid exposure and ANS treatment efficacy as determined by functional lung maturation in preterm lambs undergoing ventilation. We hypothesized that the levels of total and free (unbound) betamethasone in fetal plasma would correlate with the extent of the lung maturational effects of the ANS therapy seen in each individual materno-fetal pair.

198 [METHODS]

199 *Animal work*

200 All protocols were reviewed and approved by the animal ethics committee of The University
201 of Western Australia (*RA/3/100/1636*). This was a basic science study. Animals were
202 randomized to treatments, and post-natal ventilations / analyses were performed by
203 investigators blinded to treatments (i.e. Saline or ANS Group allocation) to defend against
204 bias or confounding.

205

206 All pregnant ewes were provided from a single supplier and experiments were performed
207 during the normal breeding season. In order to prevent preterm labor, thirty date-mated ewes
208 carrying singleton fetus received an intramuscular injection of 150mg medroxyprogesterone
209 acetate (Depo-Ralovera®; Pfizer, West Ryde, NSW, Australia) at 114±1 days of gestational
210 age (term = 150 days). This treatment has been previously shown to have no influence on
211 lung maturation in sheep models.[12] Five days later all pregnant ewes underwent recovery
212 surgery to catheterize the maternal and fetal jugular veins as previously described, with
213 catheter lines exteriorized through a flank port and secured in a sterile container adherent to
214 the ewe's back as previously described.[13] Animals recovered for 24 hours before being
215 randomized to one of two groups: either **i**) a single maternal intramuscular injection of 2 ml
216 saline (Control Group, n=10); or **ii**) a single maternal intramuscular injection of 0.25 mg/kg
217 betamethasone acetate + betamethasone phosphate (Celestone Chronodose, Merck Sharp &
218 Dohme, Australia) (ANS Group, n=20) at 120±1 days of gestational age. We have previously
219 demonstrated an efficacious response from this dose in the sheep model of pregnancy. 2-3ml
220 of maternal and fetal blood was collected at 13 time points from jugular catheters over 48

hours following saline or betamethasone administrations (Collection time points: -10 minutes, 1 , 2, 4, 6, 8, 10, 12, 16, 24, 30, 36, 48 hours). Samples were centrifuged at 3000 x *g* to separate plasma, which was then frozen at -80°C for subsequent analysis. Half of the plasma at the 1, 6, 12, 24, 36, 48 hour time points was collected into Centrifree® Ultrafiltration Devices (EMD Millipore Corporation. Billerica, MA, USA) and centrifuged at 1000 x *g* for ten minutes at room temperature to separate free from protein-bound betamethasone. A pilot experiment was performed initially to show that there was no difference in free:bound betamethasone yield when extractions were performed at room temperature (23°C) or sheep core temperature (38.9°C) (data not shown). Lambs were delivered under terminal anesthesia for a 30 minute ventilation procedure at the conclusion of the 48h sampling period to test for functional lung maturation.[13]

Ventilation

At 122±1 days gestational age, pregnant ewes received an intravenous injection of midazolam (0.5 mg/kg) and ketamine (10 mg/kg) followed by a 3mL spinal injection of lidocaine (20 mg/ml) for surgical delivery. After surgical delivery, the lamb received an intramuscular injection of ketamine (10 mg/kg) and a 4.5Fr endotracheal tube was placed by tracheostomy. Lambs were then weighed, dried, and placed on a temperature controlled radiant warmer (Cosy Cot, Fisher & Paykel Healthcare, New Zealand). Mechanical ventilation using Acutronic Fabian infant ventilators (Acutronic Medical System, Hirzel, Switzerland) was started immediately and maintained for 30 minutes on the following setting: peak inspiratory pressure (PIP) of 35 cmH_2O, positive end expiratory pressure (PEEP) of 5 cmH_2O, respiratory rate of 50 breaths per minutes, inspiratory time of 0.5

seconds, and 100% heated and humidified oxygen. An umbilical artery catheter was placed to measure arterial blood pH, pO_2, pCO_2, heart rate and blood pressure during ventilation. Ventilation efficacy index (VEI) was calculated as follows: VEI = 3800 / [respiratory rate (PIP – PEEP) x pCO_2 (mmHg)].[14] The investigators that performed ventilations were blind to treatment allocation.

Necropsy

Ewes and lambs were euthanized with an intravenous lethal dose of pentobarbital. At necropsy, the lamb's chest was opened surgically to measure lung compliance with a static pressure volume curve. The lungs were then removed and weighed. The right lower lobe was dissected and frozen for molecular studies.

Measurement of transcript expression changes in the fetal lung

RNA was extracted from lung tissue (right lower lobe) using RNeasy® Plus Mini Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's instructions. The concentration of extracted ribonuclease acid was determined using a broad-range acid quantitation kit and a Qubit 2.0 fluorometer (both Life Technologies, Carlsbad, CA). All ribonuclease acid (RNA) extracts were diluted in nuclease-free water (Life Technologies) to achieve a final RNA 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) with a Step One Real-Time PCR system in accordance with manufacturer's instructions. Messenger RNA

transcripts for surfactant protein A (SP-A), surfactant protein B (SP-B), surfactant protein C (SP-C), aquaporins 1 (AQP-1), aquaporins 5 (AQP-5) and epithelial sodium channel subunits B (ENaC-B) were measured. Ribosomal protein 18s was used as internal reference to normalize the amplification data for each gene. Delta quantification cycle values were used to determine relative expression of transcripts.

Measurement of plasma free and total (free + bound) betamethasone concentration by mass spectrometry

Unfiltered plasma collected from maternal and fetal blood samples was used to analyze total (free and bound) betamethasone concentration. Filtered plasma separated by Centrifree® Ultrafiltration Devices was analyzed to measure free (unbound) betamethasone concentration.

Plasma samples and betamethasone standards (200, 100, 40, 20, 10, 2, 1, 0 ng/ml) in control fetal plasma were extracted as previously described and analyzed by mass spectrometry.[13] Quality control samples were run in duplicated across the experiment to determine assay precision. Intraassay coefficient of variation was 0.8-7.2 %. The interassay coefficient of variation was 4.8 %. Data were fitted to a 2-compartmetnt model with PKSOLVER.[15] All R^2 values for calibration curves were >0.99.

Definition of ANS Responder and Non-Responder Sub-Groups

ANS Group animals (1 x 0.25mg/kg 1:1 betamethasone phosphate + betamethasone acetate) were initially analysed as a single group to confirm overall treatment benefit before being

divided into Responder or Non-Responder Sub-Groups on the basis of arterial $PaCO_2$ levels at 30 minutes ventilation. A value of 2 standard divisions (SD) from the average of arterial $PaCO_2$ at 30minutes ventilation from Control Group animals was used as an arbitrary, *a priori* cut-off for sub-group distribution, based on our earlier work with this model system. The Responder Sub-Group was defined as arterial $PaCO_2$ level more extreme than 2SD from the Control Group mean, and the Non-Responder Sub-Group was defined as an arterial $PaCO_2$ level within 2SD of the Negative Control Group mean.

Statistical analysis

Statistical analysis was performed using IBM SPSS for Windows, version 25.0 (IBM Corp, Armonk, NY). Mean differences between parametric data were tested for significance with *t*-test or Mann-Whiney test. The Control Group was first compared with the ANS Group as a primary analysis. Thereafter, comparisons between the Responder Sub-Group and the Non-Responder Sub-Group were performed. In the Sub-Group analysis, only parameters that were identified as significantly different in the primary analysis were assessed. Comparisons of betamethasone concentration were performed by *t*-test or Mann-Whiney test. Significant was attributed to 95% confidence interval (95% CI) or P values <.05.

[RESULTS]

One animal in each of the Control and ANS groups failed to complete their treatment protocol and were removed from further analyses. Nine lambs in the Control Group and 19 lambs in the ANS Group completed their assigned protocols. The ANS Group was divided into Responder (n=12) and Non-Responder (n=7) Sub-Groups as described above. The betamethasone treatment efficacy rate was 63% based on the cut-off. There were no significant differences in gestational age, birth weight, sex, cord blood pH, cord $PaCO_2$, or lung weight at delivery between primary groups and sub-groups (Table 1).

30 minute ventilation results

Figure 1 and Supplementary Table 1 present key physiological variables and arterial blood gas measurements after 30 minutes of ventilation. Given a 30 minute $PaCO_2$ for Control Group of 129.8±19.4 mmHg, the $PaCO_2$ cut-off for determining ANS response was 91.0 mmHg. Only one animal in the Control Group had a 30 minute $PaCO_2$ lower than the arbitrary cut-off (89.6 mmHg) used for sub-group analyses. As this animal was not statistically an outlier it was retained for subsequent analyses.

At primary analysis, there were significant increases in pH and PaO_2 in the ANS Group relative to the Control Group. $PaCO_2$ and heart rate decreased significantly in the ANS Group compared to the Control Group. There was no significant difference in mean blood pressure between the Control Group and the ANS Group. For Sub-Group analyses, pH, PaO_2, $PaCO_2$ and heart rate were analysed.There were significant increases in pH and PaO_2 and significant decreases in $PaCO_2$ and HR after 30 minutes ventilation in the Responder Sub-Group

compared to the Non-Responder Sub-Group.

Tidal volume and VEI after 30 minutes ventilation was significantly improved in the ANS Group relative to the Control Group while there was no difference in dynamic compliance (Figure 2). Sub-Group analysis showed that both tidal volume and VEI were increased significantly in the Responder Sub-Group compared with the Non-Responder Sub-Group. Dynamic compliance was not analyzed in Sub-Group analysis. Animals in the ANS Group had significantly improved static lung compliance compared with the Control Group at primary analysis. The Responder Sub-Group showed a significant improvement in static lung compliance compared with the Non-Responder Sub-Group, as demonstrated by the pressure-volume curve and lung gas volume at 40 mmH_2O (Figure 3). There was no evidence of gross lung injury in any of the groups.

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

At primary analysis, relative to the Control Group, significant increases were detected in the relative expression of transcripts for SP-A, SP-C, AQP-1, AQP-5 and ENaC-B in the ANS Group while there was no significant differences in SP-B between the Control Group and the ANS Group. At Sub-Group analysis, there was no significant difference in the relative expression of transcripts between the Responder Sub-Group and Non-Responder Sub-Group. (Figure 4).

Total and free betamethasone measurements

Total betamethasone concentration in maternal and fetal plasma was analyzed at 13 serial time points. Free betamethasone concentration was analyzed at 6 serial points. Maternal and fetal exposure to betamethasone was calculated by determining area under the curve. There were no significant differences in maternal and fetal total or free betamethasone, the betamethasone half-life, maximum concentration time, maximum concentration and area under the curve between the Responder Sub-Group and the Non-Responder Sub-Groups (Figure 5, Supplementary table 2).

[COMMENT]

Principal Findings

This study showed that animals in the ANS group (receiving one clinical dose of ANS treatment - 0.25mg/kg betamethasone phosphate and acetate) had improved lung maturation physiologically at 48 hours after the treatment relative to animals in Control Group which were treated with saline. Observed treatment efficacy was variable, with ~40% of preterm lambs failing to respond to treatment. ANS Group Responder and Non-Responder Sub-Groups could established based on an $PaCO_2$ levels after 30 minutes of ventilation.

Among animals in the ANS Group, there was no difference in betamethasone pharmacokinetics, including protein binding (i.e. free:bound betamethasone in plasma) between the Responder Sub-Group and the Non-Responder Sub-Group. These data suggest that, at least in the sheep model of pregnancy, observed variation in standardized ANS treatments are likely due to pregnancy or fetus-specific factors impacting how the fetus responds to an otherwise comparable glucocorticoid exposure. We hypothesized that variability in ANS treatment efficacy in previously observed in experiments involving the sheep model of pregnancy could be explained, at least in part, by differences in individual clearance and distribution of betamethasone in the maternal and fetal compartments. This hypothesis was not supported by our study findings.

The initial primary analysis showed that ANS treatment improved lung maturation as demonstrated by blood gas analysis and ventilation analysis. Moreover, ANS promoted alterations in mRNA expression which were consistent with lung maturation. These changes

were similar to those seen in our previous experiments with this model system. [16]

Sub-Group analysis showed ANS efficacy in just 60% of treated animals. An arbitrary cut-off value 2 SD lower than average of $PaCO_2$ at 30minutes ventilation in the control group was an appropriate means of interrogative ANS responsiveness based on the experimental design. Other markers, such as PaO_2 may not have been such a robust maker of lung maturation, because of variation in ductus arteriosus in size and flow direction may have impacted values.[17]

After administration, drugs have the potential to bind to serum in the blood. In particular, betamethasone predominantly binds to serum albumin or alpha-1-acid glycoprotein (AGP). It is believed that free drugs, not bound to protein, are better able to traverse biological membranes of cells and exert their pharmacological effects.[18, 19].

We used Centrifree Ultrafiltration Devices with a nominal molecular weight limit of 30 kDa. Considering albumin and AGP have a molecular weight greater than 30 kDa, we suggest that this approach provides a robust means of easily separating free betamethasone from bound betamethasone.[20] While physiological and molecular results in the ANS Responder Sub-Group were significantly different from those of the ANS Non Responder Sub-Group, no differences (particularly in drug half-life and AUC), were identified between these groups. Therefore, we concluded that our hypothesis was incorrect, and that the observed variation in ANS treatment efficacy was not due to betamethasone clearance, plasma protein binding, or distribution.

Clinical Implications

Although ANS therapy has been widely adopted, the dosing strategy employed has remained largely unoptimized for some 50 years. Additionally, it is important to recognize that a significant percentage of human fetuses appear to be similarly non-responsive to ANS treatment. From a clinical standpoint it is important to consider that, despite effectively controlling for gestational age, plurality, dose, agent and time to delivery interval, only ~60% of animals in the ANS Group in this study responded positively to treatment. It is unclear, given the range of factors involved, if such control can be achieved in a clinical setting, and this may be reflected in the apparently lower rate of benefit; based on Cochrane Review, ANS reduced RDS by 40 %.[6] A significant percentage of this variation will almost certainly be due to factors such as agent used and treatment to delivery interval; however it seems reasonable to suggest, based on evidence of variability in adult responsiveness to steroid treatments, and data presented herein, that a meaningful percentage variation of human preterm response to ANS therapy likely derives from individual differences in steroid responsiveness - rather than drug exposure *per se*.[21]

This is an important avenue of future investigation given both the potential benefits deriving from appropriate ANS application, and the risks conveyed by off-target use, with repeated courses of ANS having received particular attention. [8, 22] In some centers, more than half of the preterm babies treated with ANS are born outside the proposed ANS treatment efficacy window (24 hours to 7 days post administration), because predicting the timing of preterm labor precisely and judging the timing of ANS therapies adequately is often difficult.[23, 24]

Strengths and Limitations

In assessing these data, it is important to reiterate that they were generated in a sheep model of pregnancy using a small number (relative to a clinical efficacy study) of surgically manipulated animals. The direct translatability of these data to the human clinical scenario remains to be established; however, the sheep has to date proven a high-fidelity model for the development of ANS therapy, and a similar degree of variability in treatment efficacy is seen in humans. Our study design incorporates a sub-group analysis, which may increase the risk of a type II error; to defend against this we have performed a main analysis (Control vs ANS Groups) to confirm overall effect. We then performed sub-group analyses only on relevant physiological changes that were statistically significant in the main analysis. Testing these findings in a clinical setting would require significantly larger numbers of subjects than used herein before a conclusive result could be obtained.

It is unknown whether lambs in the Non-Responder Sub-Group could have developed a degree of functional lung maturation if they had been subjected to a subsequent course of ANS therapy - although it was not possible to assess this point given the study design used. However, considering that the variability of ANS efficacy between the Responder Sub-Group and the Non-Responder Sub-Group was not due to betamethasone distribution, clearance or protein binding ratio it is tempting to speculate that individual sensitivity, perhaps at the receptor level, to corticosteroid stimulation plays a key role in determining ANS treatment efficacy.

Research Implications

These data suggest that in addition to delivering a necessary dose of steroids, ANS treatment efficacy additionally depends on pregnancy or fetus-specific factors. Further studies focusing on genetics and fetal responsiveness to ANS at various gestational ages are necessary. At the same time, optimized ANS therapies to deliver a more efficient outcome with an absolute minimum risk of adverse side effects need to be studied.

Conclusion

Our data show that adequate materno-fetal steroid exposure is not the sole determinant of ANS treatment efficacy. Although these data are from a comparatively small animal study, they do highlight that the optimization of dosing regimens, with an additional focus on individual patient responsiveness, may serve as an important avenue by which the efficacy and safety of ANS therapy may be improved.

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550 [Tables]

551 **Table 1. Summary of delivery data.**

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	Control	ANS	Responder	Non-responder
n	9	19	12	7
Gestational Age (d)	122.2±0.8	122.0±0.8	122.0±0.8	122.0±0.8
Birth Weight (kg)	2.9±0.4	2.6±0.4	2.7±0.4	2.6±0.4
Sex (M/F)	4/5	11/7†	8/3†	3/4
Cord pH	7.30±0.10	7.27±0.04	7.26±0.05	7.27±0.03
Cord blood pCO2 (mmHg)	59.3±9.6	58.1±5.7	57.5±6.7	59.2±4.5
Lung Wt(g/kg)	35.3±2.4	33.9±5.1	32.9±3.9	35.9±7.2

†=Sex not recorded for one animal

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[FIGURE LEGENDS]

Figure 1. Blood gas measurements and physiological parameters

Arterial blood gas measurements and physiological parameters at 30 minutes ventilation of preterm lamb. **A**, pH (Control vs ANS, mean difference 0.15 [95% CI: 0.03 to 0.26], Responder vs Non-Responder, mean difference -0.23 [95% CI: -0.33 to 0.12]) **B**, $PaCO_2$ (Control vs ANS, mean difference -38.0 [95% CI: -60.1 to -15.9], Responder vs Non-Responder, mean difference 50.9 [95% CI: 35.8 to 66.1]) **C**, PaO_2 (Control vs ANS, mean difference 55.6 [95% CI: 14.2 to 97.0], Responder vs Non-Responder, mean difference -68.5 [95% CI: -131.5 to -5.5]) *significant difference between groups. Error bars represent ±1 standard deviation. *CI: Confidential Interval*

Figure 2. Ventilation data

Ventilation data at 30 minutes of ventilation. **A**. There was no difference in dynamic compliance between the Control Group and the ANS Group (Control vs ANS, mean difference 0.08 [95% CI: -0.02 to 0.19]). Sub-Group analysis was not done. **B**. Tidal volume showed significant differences in both primary and Sub-Group analysis. (Control vs ANS, mean difference 1.32 [95% CI: 0.48 to 2.17], Responder vs Non-Responder, mean difference -1.51 [95% CI: -2.36 to -0.67]) **C**. Ventilation efficacy index showed significant differences in both primary and Sub-Group analysis. (Control vs ANS, mean difference 0.011 [95% CI: 0.005 to 0.016], Responder vs Non-Responder, mean difference -0.015 [95% CI: -0.021 to -0.010]) *significant difference between groups. Error bars represent ±1 standard deviation.

Vt, tidal volume; VEI, Ventilation Efficiency index

Figure 3. Lung maturation analysis.

A, Statistic lung gas volume measured at a maximal pressure of 40 cmH_2O. It showed significant differences in both primary and Sub-Group analysis. (Control vs ANS, mean difference 3.32 [95% CI: 1.23 to 5.40], Responder vs Non-Responder, mean difference -3.60 [95% CI: -6.00 to -1.21]) *significant difference between groups. Error bars represent ±1 standard deviation. **B,** Pressure-volume relationship for air inflation and deflation of the lung at necropsy. The lower line from 0 cmH_2O to 40 cmH_2O of pressure in each loop is air inflation and the higher lines from 40 cmH_2O to 0 cmH_2O of pressure are air deflation. The volume in each pressure was higher or almost same in Responder compared to Non-Responder, which means static lung compliance was higher in Responder than Non-Responder. The ANS group was not shown.

V40, volume at 40 cmH_2O; PV, pressure volume

Figure 4. Messenger RNA quantification in fold change relative to control animals.

Relative expression of mRNA transcript for surfactant protein A, B and C (SP-A, SP-B and SP-C), aquaporin 1 and 5 (AQP-1 and AQP-5) and epithelial sodium channel subunits B (ENaC-B). While all mRNA transcripts apart from SP-B were significantly increased in ANS Group compared to Control Group, there was no difference in Sub-Group analysis. *Box margins* represent 25^{th} and 75^{th} percentiles; *horizontal line* represents median value. *significant difference between groups. *Circles* represent outlier values >1.5 times the interquartile range.

Figure 5. Plasma betamethasone concentrations plotted against time.

Total (unbound + bound) betamethasone concentrations and free (unbound) betamethasone concentrations in both maternal and fetal plasma from drug administration to delivery were measured. **A,** total betamethasone concentrations in maternal plasma; **B,** free betamethasone concentrations in maternal plasma; **C,** total betamethasone concentrations in fetal plasma; **D,** free betamethasone concentrations in fetal plasma. There are no significant differences in each measurement between responder and non-responder. *Data points* represent mean group values. *Error bars* represent 1 standard deviation.