Betamethasone phosphate reduces the efficacy of antenatal steroid therapy and is associated with lower birth weights when administered to pregnant sheep in combination with betamethasone acetate.

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[**TITLE**]

Betamethasone phosphate reduces the efficacy of antenatal steroid therapy and is associated with lower birth weights when administered to pregnant sheep in combination with betamethasone acetate.

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[**CONDENSATION**]

In the present study, low dose treatment with betamethasone acetate alone achieved more consistent functional maturation of the preterm ovine lung compared to combined betamethasone acetate and betamethasone phosphate treatment, but without causing a significant reduction in fetal growth. We concluded that the elevated materno-fetal betamethasone concentration derived from the inclusion of betamethasone phosphate in antenatal corticosteroid therapy is likely redundant for fetal lung maturation, and may increase the risk of harm.

[**SHORT TITLE**]

The inclusion of betamethasone phosphate in ACS therapy was associated with increased treatment variability, lower birth weights, and did not improve lung maturation relative to use of betamethasone acetate alone.

[**AJOG AT A GLANCE**]

A. *Why was this study conducted?*

Antenatal corticosteroid (ACS) therapy is widely used to improve preterm outcomes, with the primary beneficial effect being precocious maturation of the fetal lung. Although in clinical use for over 50 years, dosing is poorly optimized and adverse effects remain of concern. We and others have previously suggested that the materno-fetal steroid exposures achieved with current clinical dosing are excessive and may increase the risk of harm. Using a sheep model of pregnancy, we studied the lung maturation and pharmacodynamic effects of betamethasone

acetate when administered individually, and in combination with betamethasone phosphate as is widely used today.

B. *What are the key findings?*

We demonstrated that two doses of 0.125 mg/kg betamethasone acetate were associated with fetal lung maturation at least as effective as a standard clinical course of two 0.25 mg/kg doses of betamethasone phosphate + acetate. Surfactant protein A expression was strongly correlated with functional maturation of the fetal lung. The ACS therapy response rate was significantly higher in the low dose betamethasone acetate-only group compared to higher dose combined betamethasone phosphate + acetate group. Maternal and cord plasma betamethasone levels were significantly higher in the combined treatment group, the elevation of which were associated with lower birth weight and a greater degree of HPA axis perturbation, consistent with the established dose-dependent response to antenatal steroids.

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

We and others have demonstrated that functional lung maturation can be achieved at exposures significantly lower than those derived from current clinical dosing protocols. This report demonstrates betamethasone phosphate is redundant when used in combination with betamethasone acetate to precociously mature the preterm lung. Our data suggest that the elevated fetal betamethasone levels deriving from use of betamethasone phosphate are associated with a greater risk of lower birth weight and a greater degree of HPA axis perturbation. Critically, these data also suggest that combining betamethasone phosphate with acetate may, relative to the use of betamethasone acetate alone, increase the risk of ACS non-responsiveness.

89 [**SELECTED FIGURE**]

90 Figure 3

91

[**ABSTRACT**]

Background: Antenatal corticosteroid (ACS) therapy is standard of care for women at imminent risk of preterm labour. Despite this, much remains to be understood regarding an optimal (maximum benefit, minimal risk of side effects) ACS dosing strategy. Although conveying overall benefit when given to the right patient at the right time, ACS treatment efficacy is highly variable, and is not risk-free. Building on earlier findings, we hypothesized that when administered in combination with slow-release betamethasone acetate, betamethasone phosphate and the high materno-fetal betamethasone concentrations it generates are redundant for fetal lung maturation.

Objective: Using an established sheep model of prematurity and post-natal ventilation of the preterm lamb, we aimed to compare the pharmacodynamic effects of a low-dose treatment with betamethasone acetate only against a standard dose of betamethasone phosphate and betamethasone acetate as recommended by the American College of Obstetricians and Gynaecologists for women at risk of imminent preterm delivery between 24 and 35+6 weeks' gestation.

Methods: Ewes carrying a single fetus at 122±1 d gestational age (term=150d) were randomized to receive either: **i)** maternal intramuscular injections of sterile saline (the Saline Negative Control Group, n=12), **ii)** two maternal intramuscular injections of 0.25 mg/kg betamethasone phosphate + acetate spaced by 24h (the Beta-P+Ac Group, n=12); or **iii)** two maternal intramuscular injections of 0.125 mg/kg betamethasone acetate spaced by 24h (the Beta-Ac Group, n=11). Fetuses were surgically delivered 48h after treatment initiation and ventilated for 30 minutes to determine functional lung maturation. Fetuses were euthanized after ventilation and lung were collected for analysis using quantitative polymerase chain

reaction and western blot assays. Fetal plasma ACTH levels were measured in the cord blood samples taken at delivery.

Results: Preterm lambs were defined as either ACS treatment responders or non-responders using an arbitrary cut-off, being a $PaCO_2$ level at 30 minutes of ventilation being more extreme than two standard deviations from the mean value of the normally-distributed Saline Control Group values. Relative to Saline Control Group animals, both ACS treatment group animals showed significantly improved lung physiological responses (blood gas and ventilation data) and had a biochemical signature (mRNA and surfactant protein assays) consistent with functional maturation. However, the Beta-Ac Group had a significantly higher treatment response rate than the Beta-P+Ac Group. These physiological results were strongly correlated to the amount of surfactant protein A. Birth weight was lower in Beta-P+Ac Group and the fetal HPA axis was supressed to a greater extent in the Beta-P+Ac Group.

Conclusion: Low dose ACS therapy solely employing Beta-Ac was sufficient for fetal lung maturation. The elevated materno-fetal betamethasone concentrations associated with the co-administration of betamethasone phosphate did not additionally improve lung maturation, but were associated with greater HPA axis suppression, a lower ACS treatment response rate, and lower birth weight – outcomes not desirable in a clinical setting. These data warrant a clinical investigation of sustained, low-dose ACS treatments that avoid high materno-fetal betamethasone exposures.

[**KEYWORDS**]

Betamethasone phosphate, Betamethasone acetate, glucocorticoid, glucocorticoid receptor, antenatal corticosteroids, surfactant protein, lamb, sheep, preterm birth, lung maturation, HPA axis.

[**Text**]

[**BACKGROUND**]

One of the most pressing challenges facing preterm babies (born before 37 weeks' gestation) is the transition to breathing room air. As such, perinatal care is significantly focussed on improving preterm lung function. [1, 2] The introduction of antenatal corticosteroid (ACS) therapy, subsequent to Liggins and Howie reporting the beneficial effects of ACS in their landmark 1972 paper, has resulted in an improved prognosis for a large cross-section of preterm born babies.[3] Today, ACS therapy is standard of care for women at risk of imminent preterm delivery. The American College of Obstetricians and Gynecologists presently recommends the use of a single course of ACS for pregnant women between 24 to 33 weeks' gestation who are at risk of preterm labor within 7 days. [4] While ACS therapy has been used widely, and benefits clearly demonstrated when given to the right women at the right time, concerns remain regarding an increased potential risk of adverse effects, including fetal growth restriction, neonatal hypoglycaemia, and negative effects on the maternal and fetal hyoithalamus-pituitary-adrenal (HPA) axis. [5-7][8] ACS efficacy *per se* has also been shown to vary between individuals in similar environments. Numerous Cochrane reviews of outcomes from a single course of ACS therapy highlight a reduction in respiratory distress syndrome of approximately 40%.[13]. A similar pattern has been observed in animal studies – both those initially published by Liggins and in more recent studies by our group, which demonstrate an ACS treatment efficacy in around 60% preterm sheep administered an accurately timed dose and ventilated under standard conditions for thirty minutes. [14].

Despite good evidence that there is room for improvement in ACS treatment efficacy, reliability, and optimal patient selection, there remains sizable geographic variation in the usage of ACS. It is clear that an optimal ACS treatment strategy is yet to be determined, and

that studies to inform a regimen to improve efficacy and durability, whilst minimising the risk of adverse effects are warranted.

The present study was undertaken with this objective in mind. We focussed on the differential pharmacodynamics of betamethasone phosphate (Beta-P) and betamethasone acetate (Beta-Ac) which are commonly used in combination as an ACS therapy. These two agents have distinct pharmacokinetic profiles after intramuscular injection.[17] Beta-P can be absorbed quickly after intramuscular administration, which leads to high peak concentration and short half-life. Beta-Ac slowly dissolves before diffusing into the vascular space. This signature enables Beta-Ac to have a far longer half-life, with a much lower maximum concentration and delayed peak concentration time. [18, 19]

We have previously demonstrated that once a low fetal plasma betamethasone threshold has been achieved (approximately 1-4ng/mL), further elevations in maternal and fetal plasma betamethasone concentrations do not additionally benefit fetal lung maturation. [20] We have also shown that a constant exposure of ≥26 hours is required for lung maturation in preterm lambs delivered 48h after ACS treatment initiation. [21] Given the evidence showing a bi-phasic glucocorticoid signalling response in key lung maturation determinants (e.g. surfactant protein A) [22], we hypothesized that the high fetal betamethasone levels achieved by the betamethasone phosphate component of combined Beta-P and Beta-Ac therapy would be redundant for driving preterm lung maturation.

To test this hypothesis, we used a preterm sheep model to explore the pharmacodynamic differences between a single course of combined Beta-P and Beta-Ac used clinically in Australia and the United States, against a single course of Beta-Ac alone.

[**Materials and Methods**]

Animal work

All protocols were reviewed and approved by the animal ethics committee of The University of Western Australia (RA/3/100/1702). All animals used were obtained from a single supplier. Experiments were performed in the same place and within a two week period during the normal breeding season. 36 date-mated ewes carrying a singleton fetus were randomized to one of three groups (n = 12 / each group): **i)** a Saline Control Group receiving maternal intramuscular saline injections only; **ii)** a single course Beta-P + Ac Group receiving two maternal intramuscular injections of 0.25 mg/kg Beta-P + Ac (Celestone® Chronodose®, Merck Sharp & Dohme, Australia) spaced by 24 hours on 121 and 122 days' gestation; or **iii)** a single course Beta-Ac Group receiving two maternal intramuscular injections of 0.125 mg/kg Beta-Ac (Hovione, Portugal) on 122 and 123 days' gestation. Each ewe received an intramuscular injection of 150 mg medroxyprogesterone acetate (Depo-Ralovera; Pfizer, West Ryde, NSW, Australia) at least five days prior to steroid or control treatments to reduce the risk of steroid-induce preterm labour (term = 150 days). This treatment has been previously shown not to influence ovine fetal lung maturation. [23] Injectable 3mg/mL solutions of active pharmaceutical ingredient betamethasone acetate (Hovione, Portugal) were prepared immediately prior to the study commencing by Oxford Compounding (Perth, Western Australia) and tested for sterility, potency and the absence of endotoxin contamination. An overview of the experimental design is provided in Figure 1. In keeping with good ethical practice, Saline Control Group animals were shared with a separate study and as such received a total of four maternal intramuscular saline injections. The administration of maternal saline injections does not alter fetal lung maturation status.

Delivery and Ventilation

All animals were delivered at 123 or 124 days' gestation, with all steroid-treated animals delivered precisely 48h after receiving their first steroid treatment. At delivery, pregnant ewes received an intravenous injection of midazolam (0.5 mg/kg) and ketamine (10 mg/kg) followed by a spinal injection of 3mL lidocaine (20 mg/mL). Lambs had a tracheostomy to insert and secure a 4.5 Fr endotracheal tube. Lambs were then delivered, weighed, dried, and placed on a temperature-controlled radiant warmer (CosyCot, Fisher & Paykel Healthcare, New Zealand). Mechanical ventilation was performed using Acutronic Fabian infant ventilators (Acutronic Medical Systems, Hirzel, Switzerland), with ventilation commencing immediately following delivery and maintained for 30 minutes, initially with the following parameters: peak inspiratory pressure (PIP) of 35 cmH_2O, positive end-expiratory pressure (PEEP) of 5 cmH_2O, respiratory rate of 50 breaths per minute, inspiratory time of 0.5 seconds, and 100% heated and humidified oxygen. Tidal volume (Vt) maintained between 7.0 and 8.0 mL/kg by adjustment of the PIP only, but with maximal PIP limited to 35 cmH_2O. An umbilical artery catheter was placed to allow measurement of arterial blood pH, pO_2, pCO_2, heart rate, and blood pressure. Ventilation data including PIP, Vt, and compliance were recorded and the ventilation efficacy index (VEI), an integrated assessment of ventilation and gas exchange, was calculated as follows: VEI = 3800/ (respiratory rate [PIP – PEEP] x $PaCO_2$ [mmHg]). [24] Noting a one day difference in gestational age at delivery between groups , fetal growth curves ($y=0.0011x^2-0.1557x+5.6144$; where x = gestational age and y = delivery weight) generated from Western Australian merino-cross sheep provided by our livestock supplier and adjusted at our facilities were used to standardize gestational age across groups.

239 *Necropsy and measurement of static lung compliance*

240 Lambs were euthanized and weighed after 30 minutes ventilation. The chest was opened to
241 allow measurement of the lung pressure-volume relationship with air inflation of the lung to a
242 pressure of 40 cmH_2O followed by deflation. The volume was standardized by lung weight.
243 The right lower lobe was dissected free and frozen for molecular analysis.

244

245 *Measurement of RNA transcript expression changes in the fetal lung*

246 Messenger ribonucleic acid (mRNA) was extracted from fetal lung tissue (right lower lobe)
247 using RNeasy Plus Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's
248 instructions. The concentration of extracted mRNA was determined by a broad range nucleic
249 acid quantitation kit and a Qubit 2.0 Fluorometer (both Life Technologies, Carlsbad, CA). All
250 mRNA extracts were diluted in nuclease-free water (Life Technologies) to achieve a final
251 mRNA concentration of 25 ng/μL.

252

253 Quantitative polymerase chain reaction (qPCR) cycling was performed with ovine-specific
254 TaqMan probe and primer sets (Applied Biosystems, FosterCity, CA) with an OneStep Real-
255 Time PCR System according to the manufacturer's instructions. The mRNA transcripts for
256 genes aquaporin 1 (*AQP-1*), aquaporin 5 (*AQP-5*), epithelial sodium channel subunit B (*ENaC-*
257 *B*), elastin (*ELN*), nuclear receptor subfamily 3 group c member 1 (*NR3C1*; also known as the
258 glucocorticoid receptor, *GR*), surfactant protein A (*SP-A*), surfactant protein B (*SP-B*),
259 surfactant protein C (*SP-C*), and surfactant protein D (*SP-D*) were measured. *AQP-1*, *AQP-5*
260 and *ENaC-B* expression is associated with improved fluid clearance in lung. A key function of
261 surfactant proteins (*SP-A, SP-B, SP-C* and *SP-D*) is alveolar stabilization and the reduction of
262 surface tension, whilst elastin plays a key role in alveolar structure and mechanical properties

under shear stress. [25, 26] 18s ribosomal protein 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.

Western Blot

20 mg of fetal lung tissue was added into 400 µL of RIPA Lysis and Extraction Buffer or T-PER Tissue Protein Extraction Reagent (both Thermo Scientific, Waltham, MA) containing cOmplete™ Protease Inhibitor Cocktail (Roche, Basel, Switzerland) at the ratio of one tablet per 10 ml of lysis buffer. Samples were prepared in Precellys 2 mL Tissue Homogenizing Mixed Bead Kit (Bertin Instrumnets, Montigny-le-Bretonneux, France) and were homogenized at 6500 rpm for 30 seconds using a Precellys 24 Tissue Homogenizer (Bertin Instruments). Samples were incubated for 90 minutes at 4°C to reduce foaming before they were centrifuged at 10,000 x *rcf* for 5 minutes. The supernatant was collected and protein concentrations were measured by Pierce Rapid Gold BCA Protein Assay Kit (Thermo Scientific).

Protein in RIPA buffer was used for glucocorticoid receptor (GR) measurements and protein in T-PER buffer was used for surfactant protein A, B and C (SP-A, SP-B, SP-C) measurements. An XCell SureLock Mini-Cell Electrophoresis System (Life Sciences) was used for electrophoresis and transfers. Samples were reduced and 15 µg or 20 µg of protein for glucocorticoid receptor (GR) assay or SP-A and SP-C, respectively, were applied to each well in NuPAGE Bis-Tris Mini Gel (Invitrogen). 20 µg of non-reduced samples were used for SP-B assay. NuPAGE 10% Bis-Tris Mini Gels were used. Electrophoresis was run for 50 minutes at 200 V constant with NuPAGE MOPS SDS Running Buffer (Invitrogen). Protein was transferred to Low-Fluorescence PVDF Transfer Membranes at 30 V constant for one hour as

per the manufacturer's protocol. Membranes were then incubated with No-Stain Protein Labelling Reagent (Invitrogen) to normalize total protein. Membranes were then incubated with Blocker FL Fluorescent Blocking Buffer (Thermo Scientific) for 30 minutes followed by overnight primary antibody incubation at 4°C. Primary antibodies were diluted into SuperSignal Western Blot Enhancer (Thermo Scientific) as follows: anti-glucocorticoid receptor antibody (ab225886, abcam, Cambridge, UK) at 1/2,000, anti-surfactant protein A/PSAP antibody (ab115791, abcam) at 1/1,000, anti-mature surfactant protein B antibody (WRAB-88912, Seven Hills Bioreagents, OH, kindly provided by Professor Jeffrey Whitsett,Cincinnati Children's Hospital, Cincinnati, OH) at 1/5,000 and anti-prosurfactant protein C antibody (ab40879) at 1/10,000. Washed membranes were incubated with Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 800 (Invitrogen) at 1/10,000 in wash buffer (phosphate-buffered saline with 0.05% Tween 20; both Sigma-Aldrich, St. Louis, MO) for 60 minutes.

Membranes were analysed using an iBright FL1000 Imaging System (Invitrogen) and target band concentrations were measured and normalized by total protein concentration. To normalize the difference between membranes, standard quality control samples were transferred to each membrane and probed. A concentration-dependent densitometry response was confirmed with serially diluted lung protein. (Supplemental figure) Thymus extracts were used as negative control samples to confirm surfactant protein band specificity.

Correlation and Regression analysis of surfactant protein and physical data

Regression analysis was performed to explore a relationship between surfactant protein expression and ventilation data. Multiple linear regression was used to predict V40 based on

311 SP-A (protein), SP-B (protein), and SP-C (protein). A simple linear regression model was used
312 to predict PaCO2 based on V40.

313

314 *Hematological analysis from Maternal and Fetal blood*

315 Maternal and fetal umbilical artery cord blood collected at delivery was assayed for plasma
316 cortisol, adrenocorticotropic hormone (ACTH), and betamethasone concentrations. Cortisol
317 and ACTH level were measured by an independent clinical pathology laboratory (Vetpath,
318 Perth, Australia). The detection limit was 5.5 nmol/L for cortisol levels and 5 pg/mL for ACTH
319 levels. For the purposes of statistical analyses, we assumed a 5 pg/mL ACTH level for samples
320 which were found to be below the limit of detection. Betamethasone concentrations were
321 measured with mass spectrometry as described previously. [14, 20]

322

323 *Definition of antenatal corticosteroid response*

324 We defined animals as either ACS treatment responders or non-responders as described
325 previously. [13, 14] Briefly, we used normally distributed Saline Control Group data and set
326 an arbitrary cut-off, based on cord arterial $PaCO_2$ levels after 30 minutes of ventilation. Those
327 animals considered to have responded to treatment (responder subgroup) were defined as
328 having a $PaCO_2$ level more extreme than 2 standard deviations (SD) below the mean value of
329 the Saline Control Group's $PaCO_2$ value. Animals were defined as being ACS treatment non-
330 responders (non-responder subgroup) when having a 30-minute ventilation $PaCO_2$ within 2
331 SDs of the Control Group mean value.

332

333

334 *Statistical analysis*

335 Statistical analysis was performed using IBM SPSS Statistics for Windows, version 25.0 (IBM
336 Corp, Armonk, NY). One-way analysis of variance (ANOVA) followed by Tukey's or Games-
337 Howell's post-hoc tests was used for multiple group comparisons as appropriate.
338 Hematological data in ACS-treated groups were tested for significance with *t*-tests or Mann-
339 Whitney *U* tests. A chi-square test was used for comparing of rate of ACS responsiveness
340 between the Beta-P + Ac Group and the Beta-Ac Group.

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[**Results**]

One animal from the Beta-Ac Group delivered before the protocol commenced (prior to steroid treatment) and was removed from further analyses. All animals from the Saline Control Group and the Beta-P+Ac Group were delivered at 123 days' gestation and all of the Beta-Ac Group animals were at 124 day's gestation. There were no significant inter-group differences in sex, cord blood pH, cord blood $PaCO_2$, or lung weight per body weight. While there was no significant difference in birth weight between the Saline Control Group and the Beta-Ac Group animals, Beta-P+Ac animals has significantly lower birth weights than the Saline Control Group animals (Table 1, Figure 2). The statistically significant difference in birth weight we identified was maintained when the one-day difference in gestational age was controlled for by adjustment with the fetal growth curve. Additionally, there were no differences in birth weight between genders in each treated group (data not shown).

Ventilation Data (30 minutes)

Arterial blood gas measurements and key physiological parameters after 30 minutes of ventilation are shown in Figure 2. Both ACS treatment groups had significantly improved pH and $PaCO_2$ compared with the Saline Control Group. Only the Beta-Ac Group had a PaO_2 value significantly higher than that of the Saline Control Group. Heart rate was significantly lower in both the Beta-P+Ac and Beta-Ac Group relative to the Saline Control Group, but there were no significant differences in mean blood pressure between groups.

Based on a Saline Control Group $PaCO_2$ mean and a standard division after 30 minutes ventilation (117.8±20.8 mmHg, respectively), we set an arbitrary cut-off for determining ACS responder and non-responder animals at 76.1 mmHg. Accordingly, 33.3 % (n = 4/12) of animals in the Beta-P+Ac Group were classified as responders, compared with 81.8 % (n =

9/11) of animals in the Beta-Ac Group (Figure 3). The responder rate was significantly higher in the Beta-Ac Group relative to the Beta-P+Ac Group ($p = 0.036$). There were no animals from the Saline Control Group assigned to the responder subgroup.

Ventilation data collected 30 minutes into the procedure are presented in Figure 4. There were no differences in PIP between the ACS-treated groups. Both the Beta-P+Ac and Beta-Ac Group showed significantly higher dynamic compliance, Vt, and VEI compared with the Saline Control Group. There were no differences in those parameters between the Beta-P+Ac and Beta-Ac Group. Figure 5 shows static lung compliance during necropsy. As it showed in ventilation data, lung gas volume at 40 mmH_2O was significantly higher in the Beta-P+Ac and Beta-Ac Group than the Saline Control Group. PV curve showed both treatment groups had larger volume loops than the Saline Control Group.

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

AQP-1 and *AQP-5* mRNA transcripts were significantly elevated in the Beta-P+Ac Group compared to the Saline Control Group. There were no significant differences in these values between the Beta-Ac and Saline Control Group (Table 2). Both steroid treatment groups had significant increases in transcripts for *ENaC-B, SP-A, SP-B, SP-C* and *ELN* compared to the Saline Control Group. The Beta-P+Ac Group showed higher fold change in *ENaC-B* than the Beta-Ac. *GR* transcript levels were significantly lower in the Beta-Ac Group relative to both the Saline Control and Beta-P+Ac Groups. There was no significant difference in *GR* transcript levels between the Control Group and the Beta-P+Ac Group. *SP-D* was not different between groups.

Western Blot analysis of lung tissue

GR, SP-A, mature SP-B and pro SP-C bands were confirmed at 90 kDa, 35-37 kDa, 18 kDa, and 19 kDa, respectively (Supplemental figure). Band volumes were determined to be proportional to total protein concentration. SP-A is highly heterogenous on immunoblotting analyses due to the presence of multiple glycosylation and acetylation sites and multimeric forms resistant to reduction. [27] In the present analysis, SP-A was detected in separate bands at 35 kDa and 37 kDa. We recognized both bands as SP-A-specific due to their absence in protein extracts from ovine thymus, which does not express SP-A at discernible levels. [28] As the 37 kDa SP-A band volume was unaffected by ACS, we have only reported band volumes for the 35 kDa SP-A form in this paper. SP-B showed two bands at 18 kDa and 25kDa. As the band at 18 kDa is a homodimer of mature SP-B and the band at 25 kDa is likely proSP-B, only mature SP-B was analyzed. [29]

GR protein concentration was significantly lower in the Beta-P+Ac Group compared with the Saline Control Group. There was no difference between the Saline Control Group and the Beta-Ac Group (Table 2). Both the Beta-P+Ac Group and the Beta-Ac Group showed significantly high SP-A and pro SP-C concentrations compared to the Control Group. There were no differences between the Beta-P+Ac Group and Beta-Ac Group for GR, SP-A or pro SP-C concentrations. Only the Beta-P+Ac Group showed significant higher SP-B concentrations than the Saline Control Group.

Regression analysis of surfactant protein and physical data

Figure 6A and 6B shows the predicted correlations between SP-A (protein) concentration and V40, and between V40 and 30-minute cord arterial blood $PaCO_2$ values. While SP-A (protein) and SP-B (protein) were significant predictors of V40 (SP-A: $\beta = 0.66$, $p<0.001$, SP-B: $\beta =$

427 0.23, $p<0.05$), SP-C (protein) was not ($\beta = 0.04$, $p=0.72$) ($R^2 = 0.734$). V40 was a significant
428 predictor of $PaCO_2$ ($\beta = -0.82$, $p<0.001$, $R^2 = 0.683$). (Figure 6C). A single regression analysis
429 was calculated to predict V40 based on SP-A (protein). It showed that SP-A (protein) was
430 significant predictor of V40 ($\beta = 0.84$, $p<0.001$, $R^2 = 0.719$). (Figure 6A).

431

432 *Hematological analyses of Maternal and Fetal blood*

433 Maternal and fetal plasma levels of cortisol and ACTH were measured with maternal blood
434 and umbilical cord blood collected at delivery. Cortisol levels were below the limit of detection
435 (5.5 nmol/L) in all but three maternal blood samples from the Beta-Ac Group (16.7, 23.2, 80
436 nmol/L) and one fetal blood sample from the Beta-Ac Group (11 nmol/L); accordingly, group
437 differences in cortisol levels were not analysed. Plasma ACTH levels are shown in Figure 7A.
438 ACTH levels in two maternal samples and one fetal sample from the Beta-P+Ac Group, and
439 one maternal sample from the Beta-Ac Group were below the limit of detection at 5 pg/mL.
440 We arbitrarily replaced them with a value of 5 pg/mL to allow analysis as outlined above. No
441 difference was seen in maternal ACTH values between the Beta-P+Ac and Beta-Ac Group.
442 Fetal plasma ACTH levels were significantly lower in the Beta-P+Ac Group than the Beta-Ac
443 Group. Figure 7B shows betamethasone concentrations in maternal and fetal plasma at delivery.
444 Maternal betamethasone concentrations were six times lower and fetal concentrations three
445 times lower in the Beta-Ac Group relative to concentrations in the Beta-P+Ac Group.

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[**COMMENT**]

Principal findings

The primary findings of this study are: **i)** that a single course (two doses at 0.125 mg/kg) of betamethasone acetate achieved more consistent functional maturation of the ovine preterm lung compared to that achieved when betamethasone acetate was administered in combination with betamethasone phosphate as two 0.25mg/kg doses; and **ii**) that relative to the use of betamethasone acetate alone, a single course of combined betamethasone phosphate and acetate resulted in higher maternal and fetal plasma betamethasone concentrations in association with a greater degree of fetal HPA axis suppression and statistically significant reductions in birth weight. On the basis of these observations, it may be concluded that for deliveries occurring 48h after treatment initiation, not only does the co-administration of betamethasone phosphate with acetate fail to additionally benefit fetal ovine lung maturation, it may in fact suppress GR-driven maturational signalling in the lung, relative to that elicited by the sole administration of betamethasone acetate at a lower total dose. Based on the pharmacokinetics and mode of action of the agents used, the root cause of these differences in treatment outcomes is likely the elevated materno-fetal betamethasone concentrations derived from the use of betamethasone phosphate. Additional studies with a specific focus on molecular mechanisms of GR signalling are necessary to validate this theory.

Overall, both treated groups had improved lung maturation compared to the Saline Control Group. Favourable arterial blood gas data (pH, PaO_2, and $PaCO_2$), ventilation data (dynamic compliance, Vt, and VEI) and static compliance (V40 and pressure-volume curves) data all demonstrate that both ACS regimens could mature the preterm lung structurally, leading to more efficient gas exchange. Heart rate was also significantly reduced in both treated groups,

suggesting that both ACS therapies could stabilize the cardiovascular system, potentially by improving cardiac performance and reducing vascular permeability. Evidence for preterm lung maturation independent of betamethasone phosphate use is also provided by our mRNA transcript analyses. In the present study, both ACS-treatment groups showed significantly increased mRNA transcript for *ENaC-B*, *SP-A*, *SP-B*, *SP-C*, and *ELN* relative to Saline Control Group animals. However, there were no differences in these mRNA transcripts apart from *ENaC-B* and *GR* between ACS-treatment groups. Although there were no significant differences, these values seem to be correlated with the total amount of ACS administered, as shown in the case of *ENaC-B*. Other than for SP-B, these differences in mRNA expression did not equate to increased protein expression.

Although the changes in $PaCO_2$ values between the ACS-treatment groups were not significantly different, when animals were classified into ACS responders and non-responders on the basis of an arbitrary cut-off, derived from Saline Control Group values there was a clear difference in the inter-animal variability of the two steroid regimens. It is important to note that $PaCO_2$ levels were used instead of PaO_2 levels (which were significantly different between ACS-treatment groups) due to the potential for PaO_2 values to be confounded by alterations in the patency of the *ductus arteriosus*. [14, 30] Although not directly comparable with a clinical outcome such as respiratory distress syndrome, it is interesting to note that a significant degree of ACS non-responsiveness is regularly reported in human randomised control trials of ACS therapy, even when other variables (i.e. successful administration of treatment course, delivery within 7d of treatment, etc) are controlled for. It would be of significant interest, and great potential importance, to determine if a constant, low-concentration materno-fetal ACS exposure (either via use of betamethasone-acetate only or another appropriate regiment)

similarly reduced the variability of treatment efficacy, yielding more favourable number needed to treat values for outcomes including perinatal death and respiratory distress syndrome.

In seeking to explore the basis for the difference in ACS outcomes identified in this study, it is important to explore the pharmacokinetics and pharmacodynamics of betamethasone acetate and betamethasone phosphate. It is well known from both animal and human studies that adverse ACS effects have a clear dose-dependent risk profile, including for risks of fetal growth restriction, impairment of HPA axis function, and neurodevelopmental effects. [31-34] Given that lower materno-fetal steroid exposures are desirable, it is remarkable that the Beta-Ac Group, which received a much lower dose of glucocorticoids had lung maturation that was at least as good as that seen in the higher dose Beta-P+Ac Group. Although data are limited to effects within 48 hours of ACS treatment, it is apparent that the lower dose ACS caused less disruption to fetal growth and the HPA axis.

Dissecting the differences in the Beta-P+Ac and Beta-Ac treatment protocols that contribute to the difference in treatment effects and observed adverse outcomes in this study is of particular importance. It is important to note that the Beta-P+Ac Group not only received a much larger total dose of betamethasone, but also that this treatment conveyed a substantially higher materno-fetal plasma betamethasone concentration. Due to its high solubility, the phosphate ester of betamethasone generates a higher peak concentration (around 5 times that of Beta-Ac alone in the sheep) that is rapidly cleared. [19] Our previous results have shown that a sustained, low-magnitude betamethasone exposure is far more effective in maturing the preterm ovine lung than a brief, high exposure pulse of betamethasone. [20, 21, 35]

Clinical implications

This work has two important implications for clinical ACS use. The first relates to efforts to improve the safety and efficacy of combined betamethasone acetate and betamethasone phosphate therapy based on the protocol used by Liggins and Howie in their landmark clinical trial that is now widely employed in the United States, Australia and parts of Europe.[3] The benefits of combined therapy, when administered to the right women at the right time, are clearly supported by multiple randomised control trials.[13] However, these same data also make it clear that treatment responsiveness is extremely variable, and a number of additional studies have reported an increased risk of harm in association with betamethasone acetate and betamethasone phosphate use. [6-10] The treatment variability and ACS dose-dependent reduction in birth weight seen clinically in association with combined betamethasone acetate and phosphate use was also identified in the present study. Given that the exclusive use of betamethasone acetate reduced HPA axis disruption, lessened effects on fetal growth, and improved overall treatment success rate, it is reasonable to suggest that clinical studies to explore use of a betamethasone acetate-only therapy (or therapy with another glucocorticoid so delivered to replicate the constant, low-amplitude exposure given by betamethasone acetate dosing) are now justified. On the other hand, variable responsiveness to ACS treatment *per se* has not been fully understood. Once we have a better understanding of the critical mechanisms driving steroid-induced lung maturation there may be a chance to identify those likely not to respond early in pregnancy. We may also be able to tailor therapies to take these (likely) genetic differences into account.

Secondly, this work also has implications for ACS dosing regimens based around the sole use of betamethasone phosphate (UK and Japan) or dexamethasone phosphate (i.e. the widely

546 employed WHO-recommended protocol).[36] Whether administering two 12 mg doses of
547 betamethasone phosphate every 24h, or four 6 mg doses of dexamethasone phosphate every 12
548 hours, these two protocols each generate a pulsatile pattern of exogenous glucocorticoid
549 exposure, characterised by comparatively high concentration peaks (notably in the 12mg
550 betamethasone phosphate protocol) immediately after administration, rapidly followed by
551 concentration troughs immediately prior to the subsequent administration. Our earlier work has
552 demonstrated the importance of a constant steroid exposure and that the concentration
553 threshold for an efficacious threshold is comparably low, around 1-4 ng betamethasone per mL
554 of fetal plasma, and certainly much lower than the concentration peaks generated by
555 contemporary dexamethasone and betamethasone phosphate concentrations. [19, 35] Based on
556 these findings, and the new data presented herein, we suggest that a betamethasone or
557 dexamethasone regimen based on frequent, lower-dose treatments (perhaps as low as 0.05
558 mg/kg) may constitute an efficacious ACS treatment regimen absent of the high concentration
559 peaks that we have shown to be both redundant for fetal lung maturation, and potentially
560 causative of harm.

561

562 *Research implications*

563 Unpacking the molecular mechanisms driving the differential treatment effects identified in the
564 present study will be of particular importance to the future optimisation of this important
565 therapy. A particular focus will likely be on why a higher materno-fetal steroid exposure
566 correlates with increased variability in treatment efficacy. The regulation of surfactant protein
567 A provides some insight into one explanation for this phenomenon. Although not essential for
568 lung function, SP-A is implicated in tubular myelin formation, in the formation of surfactant
569 films and in phospholipid cycling. [37-39] In the present study, SP-A protein rather than SP-B

or SP-C showed a strong correlation with V40 which represented static lung compliance. (Figure 6A) While both SP-A and SP-B have been shown to be essential for normal lung function, SP-A protein expression is likely quite informative in assessing ACS treatment response and lung maturation status.V40 was also correlated to $PaCO_2$ at 30 minutes ventilation. (Figure 6B)

Ballard and colleagues have previously demonstrated that SP-A is exquisitely responsive to GR-stimulation. Unlike other surfactant proteins, such as SP-B, that appear to exhibit a linear response to GR-activation, SP-A appears to have a pronounced bi-phasic response, wherein maximal transcript expression occurs at a low exogenous steroid concentration, and is then reduced (apparently via both negative feedback and increased mRNA turnover) at higher steroid concentrations. [22] Bridges and colleagues have recently demonstrated the role of GR activation in the modulation of WNT, JAK-STAT and VEGF signalling in the fetal lung, leading to matrix fibroblast differentiation and mature alveolar type I and II cell transformation. [40] It is tempting to speculate that, similar to the situation observed with SP-A responses, one or more key regulatory elements in these pathways has a 'goldilocks' response to GR signalling, where too little (in terms of both magnitude and/or duration) or too much exposure results in a sub-optimal maturation response.

Strengths and limitations

A number of limitations should be taken into account when assessing the translatability of the data presented herein. Although the sheep is an excellent translational model to study ACS therapy, it should be remembered that the data are from an animal rather than a human clinical study and sex-linked differences were not accounted for. Furthermore, this study used a small

number of animals. The study was adequately powered to explore a potential difference between ACS treatments and saline control, but was not designed to assess any (likely more subtle) differences between ACS-treatment groups. It is possible that a much larger study (group sizes of ~30) may allow for identification of treatment differences between ACS-groups, and also assist in the identification of any statistically significant difference in delivery weight between the Beta-Ac Group and the Saline Control Group.

There were two limitations of study design; as appropriate for good ethical practice and the reduction of animals used in research studies, the Saline Control Group animals were shared with a separate protocol and received four maternal saline injections on different days from the animals treated with ACS. Based on a comparison with earlier data (not shown), an additional two injections of saline do not alter fetal lung development. Secondly, there was a one-day difference in gestational age between the Beta-Ac group animals and the Saline Control / Beta-P+Ac Group animals due to limitations in our sheep mating capacity. To ensure the observed difference in birthweight was not confounded by this difference, we corrected for one day of growth using fetal weight charts previously developed by our group. In performing a correction, we found that the observed difference in weights retained statistical significance. Given this, and the strong body of evidence linking a dose-dependent relationship between fetal glucocorticoid exposure and growth restriction, we are confident that the observed difference is a function of the treatment received, rather than a small difference in gestational age.

Conclusion

We hypothesised that the high fetal betamethasone levels achieved by the betamethasone phosphate component of combined Beta-P and Beta-Ac therapy would be redundant for driving

preterm lung maturation. The results of this study support this hypothesis, and also strongly suggest that lower-dose treatment with Beta-Ac, avoiding high materno-fetal steroid exposures, is both safer and more effective than combined Beta-P and Beta-Ac therapy. These findings add further impetus to the undertaking of clinical trials to optimise the agent choice and dosing regimen for ACS therapies.

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737 [Tables]

738 Table 1

739 Summary of delivery data.

740

741 Table 1

	Control	Beta-P+Ac	Beta-Ac
n	12	12	11
Gestational Age (d)	123	123	124
Birth Weight (kg)	2.8±0.3	2.4±0.3*	2.7±0.3
Sex (M/F)	5/7	8/4	6/5
Cord pH	7.33±0.15	7.36±0.15	7.37±0.15
Cord blood PaCO2 (mmHg)	53.1±4.8	48.4±4.2	46.8±4.5
Lung Weight (g/kg)	35.6±3.8	34.3±3.7	33.3±3.2

742 *: significantly lower than the Control Group ($p<0.01$)

743

744 Table 2

745 Lung messenger RNA and protein quantification in fold change relative to control animals

746

747 Table 2

		Fold change (vs Control)		
Tissue	Target mRNA	Control	Beta-P+Ac	Beta-Ac
Lung	*AQP-1*	1.00 (0.81-1.24)	1.40 (1.11-1.78)*	1.13 (0.89-1.44)
	AQP-5	1.00 (0.72-1.39)	1.76 (1.27-2.45)*	1.35 (0.96-1.88)
	ENaC-B	1.00 (0.73-1.37)	2.34 (1.77-3.10)*	1.50 (1.13-2.00)*†
	ELN	1.00 (0.84-1.20)	2.45 (1.87-3.21)*	2.08 (1.46-2.95)*
	GR	1.00 (0.84-1.19)	0.90 (0.73-1.11)	0.70 (0.57-0.87)* †
	SP-A	1.00 (0.57-1.75)	2.63 (1.58-4.37)*	2.12 (1.26-3.57)*
	SP-B	1.00 (0.71-1.40)	1.82 (1.32-2.50)*	1.47 (1.06-2.04)*
	SP-C	1.00 (0.69-1.45)	2.25 (1.52-3.34)*	1.92 (1.28-2.87)*
	SP-D	1.00 (0.65-1.45)	1.27 (0.72-2.23)	0.78 (0.44-1.39)
Tissue	Target Protein	Control	Beta-P+Ac	Beta-Ac
Lung	GR	1.00 (0.79-1.21)	0.54 (0.31-0.76) *	0.73 (0.48-0.97)
	SP-A	1.00 (0.78-1.22)	1.85 (1.37-2.32) *	2.43 (1.93-2.94) *
	SP-B	1.00 (0.52-1.47)	2.33 (1.52-3.12) *	1.73 (1.03-2.43)
	SP-C	1.00 (0.70-1.30)	1.88 (1.36-2.39) *	1.99 (1.55-2.44) *

748 Average (95% Confidence Interval)

749 *: significant difference compared to the Control Group ($p<0.05$)

750 †: significant difference between the Beta-P+Ac and Beta-Ac Group ($p<0.05$)

751 [FIGURE LEGENDS]

752 **Figure 1. Timing of interventions in each group**

753 **i**) Saline Control Group animals received four maternal intramuscular injections of 2 ml of
754 saline at 115, 116, 117 and 118 days' gestation. Fetuses were delivered at 123 days gestational
755 age. **ii**) Beta-P+Ac Group animals received two maternal intramuscular injections of 0.25
756 mg/kg of Beta-P+Ac at 121 and 122 days' gestation. Fetuses were delivered at 123 days
757 gestational age, 48 hours after commencement of intervention. **iii**) Beta-Ac Group animals
758 received two maternal intramuscular injections of 0.125 mg/kg of Beta-Ac at 122 and 123 days'
759 gestational age. Fetuses were delivered at 124 days gestational age, 48 hours after
760 commencement of intervention. *IM: intramuscular injection, GD: gestational day.*

761

762 **Figure 2. Birth weight, blood gas measurement and physiological parameters**

763 Birth weight, arterial blood gas measurements and physiological parameters at 30 minutes of
764 preterm lamb ventilation. **A**, birth weight (Saline Control vs Beta-P+Ac, mean difference -0.43
765 [p=0.007, 95% CI: -0.75 to -0.11], Saline Control vs Beta-Ac, mean difference -0.13 [p=0.619,
766 95% CI: -0.46 to 0.20], Beta-P+Ac vs Beta-Ac, mean difference 0.30 [p=0.078, 95% CI: -
767 0.03 to 0.63]) **B**, pH (Saline Control vs Beta-P+Ac, mean difference 0.26 [p<0.001, 95% CI:
768 0.14 to 0.38], Saline Control vs Beta-Ac, mean difference 0.33 [p<0.001, 95% CI: 0.22 to
769 0.44], Beta-P+Ac vs Beta-Ac, mean difference 0.07 [p=0.420, 95% CI: -0.07 to 0.21]) **C**,
770 $PaCO_2$ (Saline Control vs Beta-P+Ac, mean difference -35.8 [p=0.001, 95% CI: -57.3 to -
771 14.3], Saline Control vs Beta-Ac, mean difference -54.0 [p<0.001, 95% CI: -76.0 to -32.0],
772 Beta-P+Ac vs Beta-Ac, mean difference -18.2 [p=0.119, 95% CI: -40.2 to 3.8]), **D**, pO_2 (Saline
773 Control vs Beta-P+Ac, mean difference 36.3 [p=0.057, 95% CI: -1.0 to 73.5], Saline Control
774 vs Beta-Ac, mean difference 117.9[p=0.014, 95% CI: 25.4 to 210.4], Beta-P+Ac vs Beta-Ac,

mean difference 81.6 [p=0.100, 95% CI: -14.2 to 177.4]), **E,** HR (Saline Control vs Beta-P+Ac, mean difference -52.2 [p<0.001, 95% CI: -78.8 to -25.5], Saline Control vs Beta-Ac, mean difference -31.5[p=0.021, 95% CI: -58.8 to -4.3], Beta-P+Ac vs Beta-Ac, mean difference 20.6 [p=0.167, 95% CI: -6.6 to 47.9]), **F**, mBP (no differences). Asterisk indicates significant difference between groups. Error bars represent ±1 standard deviation. *CI: Confidence Interval.*

Figure 3. Response rate to ACS treatment

Preterm lambs were divided into a responder group or non-responder group based on an arbitrary cut-off. The cut-off was set at 76.1 mmHg in $PaCO_2$ at 30 minutes ventilation, which was 2 standard deviations below of $PaCO_2$ average in the Control Group at 30 minutes ventilation. **A,** all animals $PaCO_2$ at 30 minutes ventilation data were plotted. The dashed line shows the arbitrary cut-off at 76.1 mmHg. The animals under the dashed line were assigned to the responder group. Animals above the line were assigned to the non-responder group. **B,** Graph shows the response rate in both ACS treated group. *The Beta-Ac Group showed a significantly higher response rate at 81.8 % than the Beta-P+Ac Group at 33.3 %. (χ^2 (1)=3.69, p=0.036).

Figure 4. Ventilation data

Ventilation data at 30 minutes. **A,** PIP (Saline Control vs Beta-P+Ac, mean difference -1.66 [p=0.163, 95% CI: -3.94 to 0.60], Saline Control vs Beta-Ac, mean difference -0.81 [p=0.087, 95% CI: -1.73 to 0.11], Beta-P+Ac vs Beta-Ac, mean difference 0.86 [p=0.620, 95% CI: -1.50 to 3.21]) **B,** dynamic compliance (Saline Control vs Beta-P+Ac, mean difference 0.22 [p=0.012, 95% CI: 0.05 to 0.39], Saline Control vs Beta-Ac, mean difference 0.30 [p<0.001, 95% CI: 0.20 to 0.41], Beta-P+Ac vs Beta-Ac, mean difference 0.08 [p=0.515, 95% CI: -0.10 to 0.27])

C, Vt (Saline Control vs Beta-P+Ac, mean difference 2.78 [p<0.001, 95% CI: 1.68 to 3.87], Saline Control vs Beta-Ac, mean difference 3.40 [p<0.001, 95% CI: 2.46 to 4.35], Beta-P+Ac vs Beta-Ac, mean difference 0.63 [p=0.463, 95% CI: -0.69 to 1.94]) **D,** VEI (Saline Control vs Beta-P+Ac, mean difference 0.016 [p=0.045, 95% CI: 0.0003 to 0.0312], Saline Control vs Beta-Ac, mean difference 0.022 [p=0.004, 95% CI: 0.008 to 0.036], Beta-P+Ac vs Beta-Ac, mean difference 0.006 [p=0.680, 95% CI: -0.013 to 0.026]) Asterisk indicates significant difference between groups. Error bars represent ±1 standard deviation. *CI: Confidence Interval; PIP, peak inspiratory pressure; VEI, ventilation efficiency index; Vt, tidal volume.*

Figure 5. Lung maturation analysis.

A, Static lung gas volumes measured at a maximal pressure of 40 cmH_2O. (Saline Control vs Beta-P+Ac, mean difference 726 [p=0.001, 95% CI: 358 to 1092], Saline Control vs Beta-Ac, mean difference 1103 [p<0.001, 95% CI: 805 to 1401], Beta-P+Ac vs Beta-Ac, mean difference 377 [p=0.095, 95% CI: -56 to 812]) **B,** Pressure-volume relationship for air inflation and deflation of the lung at necropsy. The higher line from 0 cmH_2O to 40 cmH_2O of pressure in each loop is the inflation arm and the lower line from 40 cmH_2O to 0 cmH_2O of pressure is the deflation arm. Asterisk indicates significant difference between groups. Error bars represent ±1 standard deviation. *CI: Confidence Interval.*

Figure 6. Relationship between surfactant protein and lung physical maturation.

A, Graphs show correlations between protein amount of SP-A and V40 as static lung compliance. Each group was plotted with different colour. **B,** Graph shows correlation between V40 and $PaCO_2$ at 30 minutes ventilation. Each group was plotted with different colour. **C,** Structural equation modelling from surfactant proteins to lung functional maturation. The

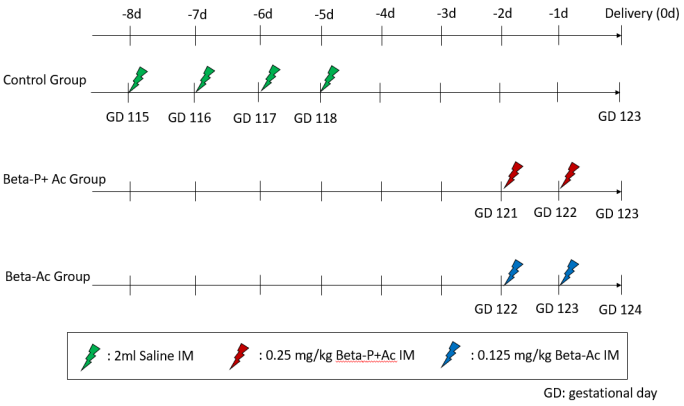
observed variables were presented by square box and latent or unmeasured variables were presented by circle. A number along with each arrow is the standardized partial regression coefficient.

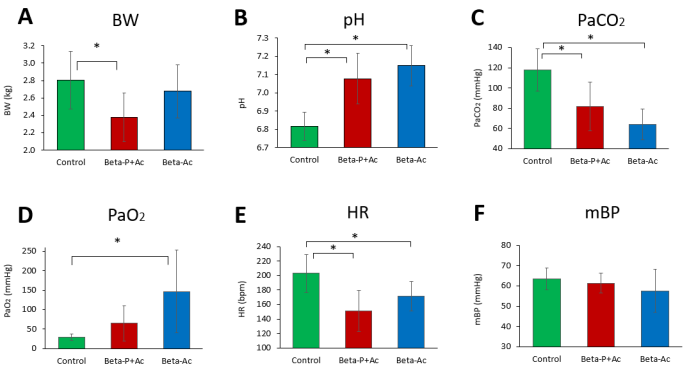
Figure 7. Hematological results from Maternal and Fetal blood

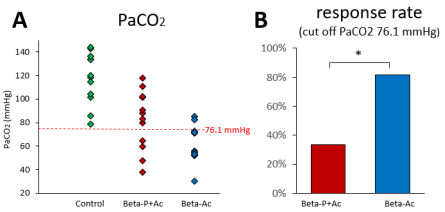
ACTH and betamethasone concentrations in maternal and fetal plasma at delivery **A**, maternal or fetal plasma ACTH levels. Three samples (two in the Beta-P+Ac Group and one in the Beta-Ac Group) from maternal plasma and one sample from fetal plasma in the Beta-P+Ac Group showed too low ACTH level to be detected. Maternal ACTH (no difference, p=0.104, Mann-Whitney U test), fetal ACTH (Significant difference, p=0.002, Mann-Whitney U test). **B**, maternal or fetal betamethasone concentration at delivery. Maternal betamethasone concentration (Beta-P+Ac vs Beta-Ac, mean difference -5.73 [p<0.001, 95% CI: -7.63 to 3.83], t-test), fetal betamethasone concentration (significant difference, p<0.001, Mann-Whitney U test). Parenthesis indicates the number of samples which could not detect ACTH. Asterisk indicates significant difference between groups. Error bars represent ±1 standard deviation. *CI: Confidence Interval*.

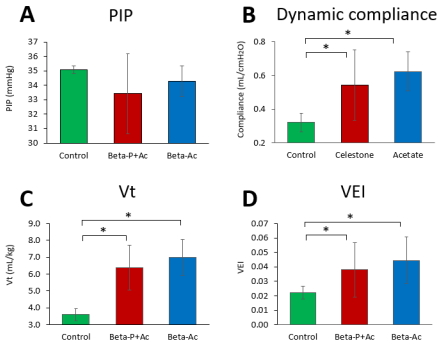
Supplemental Figure. Images of correlations between target bands and total protein amount in western blot analysis.

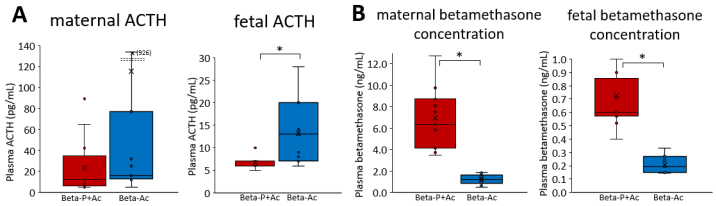
Serially diluted lung proteins every 5 μg from 5 μg to 25 μg were analysed with GR, SP-A, SP-B, and pro SP-C antibody. Thymus was also used as a negative control for SP-A, SP-B, and pro SP-C antibody. Blue bands show total protein and target bands are shown as green bands at arrows or a square bracket. Gray scale bands show target bands. Band volume of both total protein bands (blue) and target bands (green) were correlated to the amount of applied protein.

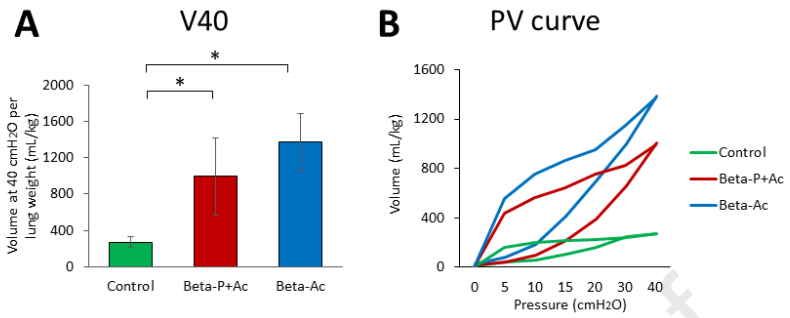


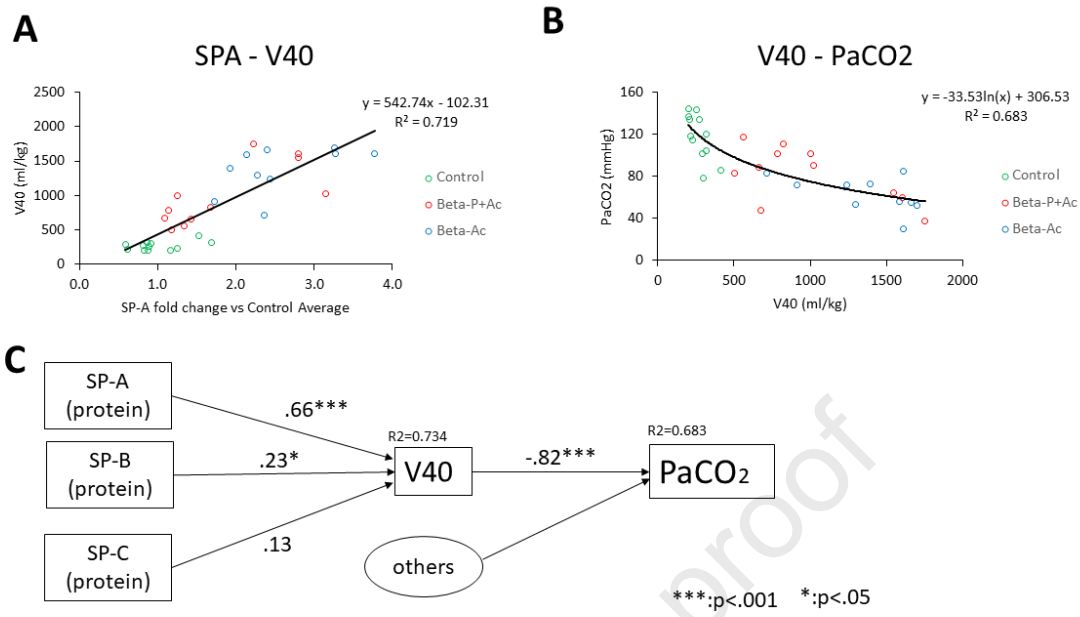


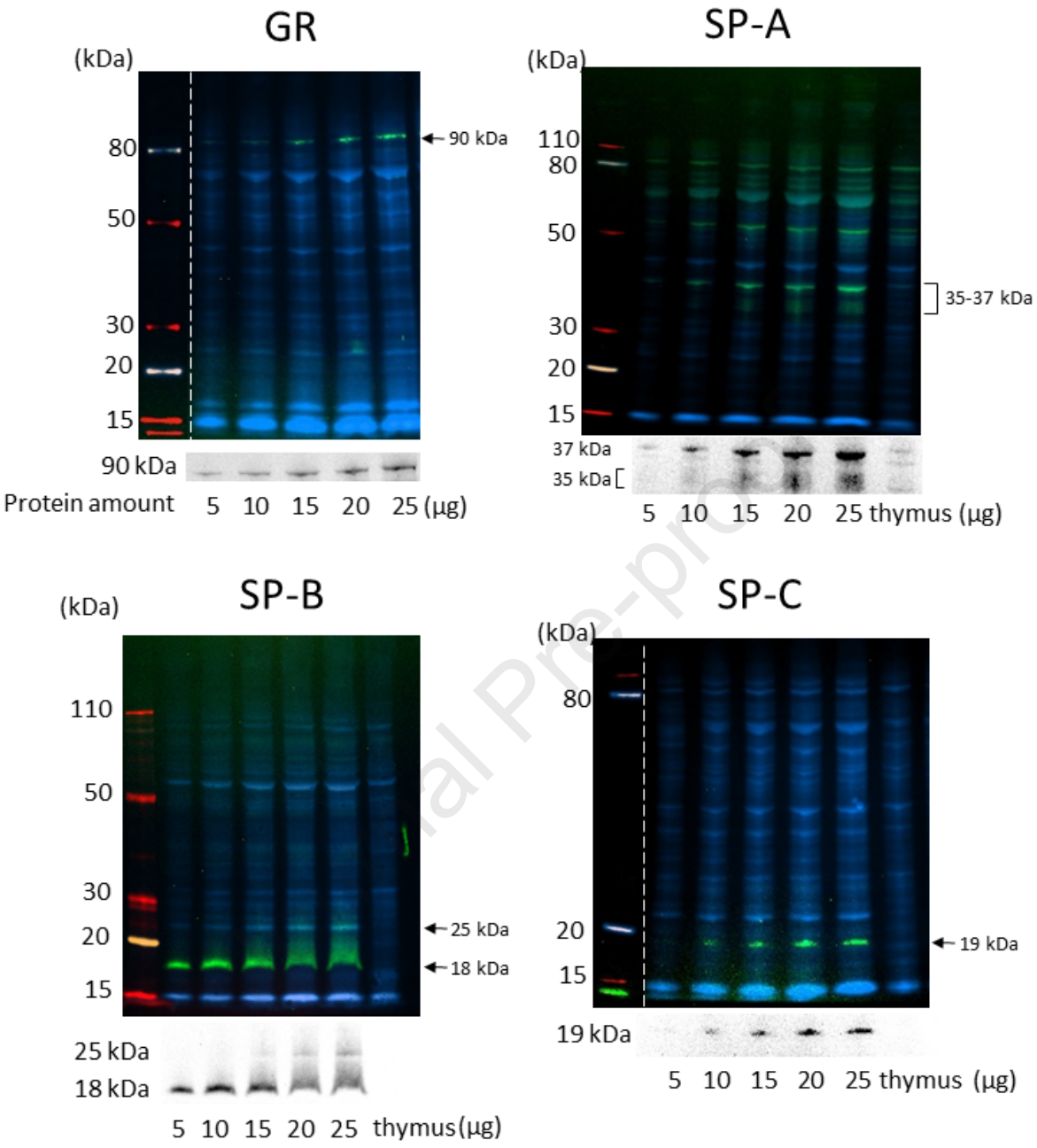








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Manuscript title:

Betamethasone phosphate reduces the efficacy of antenatal steroid therapy and increases the risk of fetal growth restriction when administered to pregnant sheep in combination with betamethasone acetate.

Corresponding author: Tsukasa TAKAHASHI MD

Authors may either sign the same form or submit individually

I am an author on this submission, have adhered to all editorial policies for submission as described in the Information for Authors, attest to having met all authorship criteria, and all potential conflicts of interest / financial disclosures appears on the title page of the submission.

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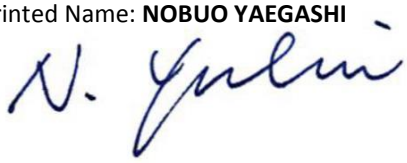

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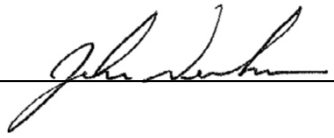

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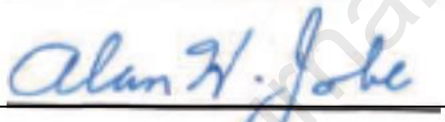

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