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## Dose of budesonide with surfactant affects lung and systemic inflammation after normal and injurious ventilation in preterm lambs

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### Abstract

**Background:** The addition of budesonide (Bud) 0.25 mg/kg to surfactant decreased the lung and systemic responses to mechanical ventilation in preterm sheep, and the rates and severity of bronchopulmonary dysplasia (BPD) in preterm infants.

**Hypothesis:** Lower budesonide concentrations in surfactant will decrease injury while decreasing systemic corticosteroid exposure.

**Methods:** Preterm lambs received either: 1) protective tidal volume ( $V_T$ ) ventilation with surfactant from birth or 2) injurious  $V_T$  ventilation for 15 minutes then surfactant treatment.

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**Consent:** No human subjects were used in this report.

Lambs were further assigned to surfactant mixed with: i) Saline, ii) Bud 0.25 mg/kg, iii) Bud 0.1 mg/kg, or iv) Bud 0.04 mg/kg. All lambs were then ventilated with protective  $V_T$  for 6 hours.

**Results:** Plasma Bud levels were proportional to the dose received, and decreased throughout ventilation. In both protective and injurious  $V_T$  ventilation, less than 4% of Bud remained in the lung at 6 h. Some of the improvements in physiology and markers of injury with Bud 0.25 mg/kg were also found with 0.1 mg/kg, whereas 0.04 mg/kg had only minimal effects.

**Conclusion:** Lower doses of Bud were less effective at decreasing lung and systemic inflammation from mechanical ventilation. The plasma Bud levels were proportional to dose given and the majority left the lung.

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## INTRODUCTION

Lung inflammation and airway injury from mechanical ventilation of very preterm infants contribute to the pathogenesis of bronchopulmonary dysplasia (BPD) (1, 2). Nearly 50% of extremely preterm infants, who are at the highest risk for BPD, will require intubation within the first few days of life (3). In preterm surfactant-deficient sheep, mechanical ventilation with both normal and injurious tidal volume ( $V_T$ ) ventilation was associated with lung inflammation and systemic changes in the liver and the brain (4–6). Corticosteroids can decrease lung inflammation and decrease the rates of BPD (7, 8). Unfortunately, widespread use of high-dose systemic dexamethasone to decrease BPD has been associated with abnormal neurologic outcomes and its use is now limited to the infants at the highest risk for BPD (8, 9). Recent clinical studies have focused on exposing the preterm lung directly to budesonide, a potent corticosteroid used to treat lung inflammation in asthma (10–12).

In preterm infants, budesonide combined with surfactant treatment at birth reduced BPD by 20% without increased mortality or adverse physiologic, neurologic, or cognitive outcomes (11–13). In an observational study of preterm infants with less severe respiratory distress syndrome, the addition of budesonide to surfactant was associated with decreased need for continued ventilation and decreased severity of BPD (14). Aerosolized budesonide primarily remains in the airways, whereas the surfactant distributes the budesonide to the distal lung (15, 16). Budesonide is then primarily retained in lung tissue as budesonide esters for delayed glucocorticoid release in children and adults, but this pharmacokinetic property has yet to be fully demonstrated in preterm infants or preterm sheep (17–19). We recently demonstrated high systemic levels of budesonide within 15 minutes of treatment with surfactant combined with budesonide in preterm sheep, and less than 2% of the budesonide remained within the lungs by 24 hours (4, 20). In the clinical studies of intra-tracheal budesonide, the plasma levels were only slightly lower than in sheep (21–23). The budesonide that enters the systemic circulation is normally rapidly metabolized to inactive products by CYP3A enzymes in the liver to minimize systemic exposures, but these enzymes may be less developed in premature infants (24, 25).

The budesonide dose (0.25 mg/kg) used in the clinical studies by Yeh *et al.* was designed to have the highest concentration of corticosteroid that would not affect the surface tension reducing properties of surfactant (11, 12, 16, 26). This dose has been effective in reducing

the lung injury and inflammation in preterm sheep ventilated with normal and injurious mechanical ventilation (4, 6). We have recently demonstrated that budesonide 0.25 mg/kg with surfactant is systemically absorbed, and alters hundreds of genes in the liver and brain of the lambs (6). Since budesonide is systemically absorbed in preterm sheep and has an affinity about 10 times higher than dexamethasone for the glucocorticoid receptor, there is concern about some of the negative systemic effects seen with early dexamethasone use for the prevention of BPD (8, 27). Lower doses of budesonide could provide similar beneficial effects in the lungs, but decreased systemic effects. We tested the pharmacology effects of lower doses of budesonide (0.1 mg/kg and 0.04 mg/kg) mixed with surfactant on lung and systemic injury from six hours of mechanical ventilation with protective and injurious tidal volume ( $V_T$ ) ventilation at birth.

## METHODS

### Animal Management.

All animal experiments were performed with the approval of the Animal Ethics Committee of the University of Western Australia. To conserve animals, some animals from previous experiments were used for comparisons (6). Additional animals were added to these groups to assure similarities between breeding periods.

Date-mated Merino Ewes at  $126 \pm 1$  days gestational age (GA; term is about 150 days GA), which had not received antenatal steroids, were anaesthetized for laparotomy and hysterotomy (4, 6). Immediately before delivery the lamb was given ketamine 10 mg/kg IM, then a 4.5 mm endotracheal tube was secured in the trachea before gentle aspiration of fetal lung fluid (FLF). The lambs were then delivered, weighed, and placed under a radiant warmer. The lambs were assigned to either an initial 15 minutes of ventilation with 1) protective  $V_T$  or 2) injury  $V_T$  (described below) and treatment with i) surfactant plus Saline, ii) surfactant plus budesonide 0.25 mg/kg, iii) 0.1 mg/kg, or iv) 0.04 mg/kg. The numbers of lambs per group (n=5 to 7) were determined from previous experiments based on multiple markers of injury or inflammation in the lung and liver (20). Unventilated lambs, euthanized at delivery, were used as controls (n=5).

### Surfactant and budesonide treatment

Lambs were assigned to receive either: i) 200 mg/kg surfactant (Poractant alfa, Curosurf® 2.5 mL/kg, Chiesi Farmaceutici, Italy) + 0.5 mL/kg Saline (Saline); ii) 200 mg/kg surfactant + 0.25 mg/kg budesonide (0.5 mg/mL, Pulmicort®, AstraZeneca, USA) (Bud 0.25 mg/kg), iii) 200 mg/kg surfactant + 0.10 mg/kg budesonide (Bud 0.1 mg/kg), or iv) 200 mg/kg surfactant + 0.04 mg/kg budesonide (Bud 0.04 mg/kg). For surfactant and budesonide dosing, 3 kg was used as the estimated fetal weight. Surfactant was gently mixed with budesonide and then administered through the endotracheal tube with body positioning to assist with distribution to the lungs (4). Timing of surfactant was dependent of ventilation group: protective  $V_T$  or injurious  $V_T$ .

### Protective $V_T$ Mechanical Ventilation

In an attempt to protect the lung and minimize lung injury, the surfactant treatments were given prior to initiation of ventilation(6). Ventilation was then begun with a Fabian ventilator (Acutronic, Switzerland) with an initial peak inspiratory pressure (PIP) of 30 cmH<sub>2</sub>O, a positive end expiratory pressure (PEEP) of 5 cmH<sub>2</sub>O, a rate of 50 breaths/minute, and an inspiratory time of 0.5 seconds, with 40% heated and humidified oxygen to 37° C (MR850 Humidifier, Fisher & Paykel Healthcare, Auckland, NZ) (4). The PIP was adjusted (limited to a maximum of 40 cmH<sub>2</sub>O) to not exceed  $V_T$  of 8 mL/kg during the 6 hours of ventilation. The normal tidal volume of a lamb is approximately 8 mL/kg versus 4 to 5 ml/kg in a preterm infant.

### Injury $V_T$ Mechanical ventilation

Prior to surfactant treatment and with the intention of initiating lung injury, ventilation was initiated with 100% oxygen, PIP of 40 cmH<sub>2</sub>O using no PEEP, a rate of 50 breaths/minute and an inspiratory time of 0.5 seconds (6). Tidal volume/kg was monitored and the PIP adjusted up to a maximum of 50 cmH<sub>2</sub>O to achieve a target  $V_T$ /kg of 12 mL/kg by 15 minutes. After 15 minutes of ventilation, the animals were treated with assigned surfactant combination and ventilated with protective  $V_T$  for 5 hours and 45 minutes.

### Animal Monitoring and assessment

The lambs received ketamine at delivery and did not breathe spontaneously. Oxyhemoglobin saturations were maintained to be greater than 90% by continuous pulse oximetry. Each lamb was continuously monitored for temperature, heart rate, blood pressure, and received a 10 mL/kg transfusion with placental blood IV and dextrose fluids (4). Arterial blood gas measurements and plasma samples for budesonide were collected at 15 minutes, 30 minutes, and then at 1 hour intervals. The lamb was euthanized at 6 hours with pentobarbital 100 mg/kg IV.

### Tissue sampling.

Postmortem inflation and deflation pressure-volume curves were measured with stepwise changes in pressure to a maximum of 40 cmH<sub>2</sub>O (28). Tissues from the right lower peripheral lung and liver were snap frozen for RNA isolation (29). The right upper lobe was inflation fixed at 30 cmH<sub>2</sub>O with 10% formalin and then paraffin embedded (29).

### Quantitative RT-PCR.

Total RNA (tRNA) was extracted from the peripheral lung tissue of the right lower lobe and the liver using Trizol (20). Custom Taqman gene primers (Life Technologies) for ovine sequences for the epithelial sodium channel (ENaC), Interleukin 1 $\beta$  (IL-1 $\beta$ ), IL-6, monocyte chemoattractant protein-1 (MCP-1), and serum amyloid A3 (SAA3) were used. Quantitative RT-PCR was performed in triplicate with iTaq Universal mix (Bio-Rad) on a CFX Connect (Bio-Rad). 18S primers (Life Technologies) were used as the internal loading control. Fold increased were determined by  $C_q$  method (CFX manager, BioRad), and average  $C_q$  for controls were set as 1, and groups reported as fold increase over unventilated controls.

### Immunohistochemistry.

Paraffin sections (4  $\mu\text{m}$ ) of formalin fixed right upper lobe tissue were used for H&E staining, and immunohistochemistry with 1:250 mouse anti-human iNOS (BD Biosciences, USA) or no antibody (negative control) (22). Blinded slides for iNOS staining had 10 random regions/animal (40X on Zeiss Axioskop 40) manually counted and scored as 0 = no positive cells, 1 = occasional positive cells, 2 = large number of positive cells iNOS positive cells were primarily in airspaces. H&E stained slides of lungs were blinded and evaluated for airway epithelial injury, edema, hemorrhage, and inflammation (0–2 points each) (21).

### Budesonide Measurements

Budesonide was measured in the plasma and lung tissue using a previously published protocol (4, 20). Lung tissue was hydrolyzed with bovine pancreas cholesterol esterase (4). Budesonide analysis was with an Agilent Technologies 1290 Infinity HPLC system and a 6460 Series Triple Quadrupole LC/MS/MS. The limit of quantitation was 0.01 ng/mL.

### Data analysis and statistics.

Variables are presented as means  $\pm$  standard deviation (SD), and the Ventilation Efficiency Index (VEI) [ $3800/(\text{PIP} \times \text{rate} \times \text{PaCO}_2)$ ] and Oxygenation Index (OI) [ $\text{FiO}_2 \times \text{Mean Airway Pressure}/\text{PaO}_2$ ] calculated (30, 31). mRNA is reported as fold increase over control values set to 1. Statistics were analyzed with Prism 6 (GraphPad) using the Student's *t*-test, Mann-Whitney non-parametric, or ANOVA tests as appropriate. Significance was accepted as  $p < 0.05$ .

### Results

All animals assigned to the protective  $V_T$  groups survived the 6-hour ventilation period. As might be expected in extremely preterm lambs ventilated after an intentional period of lung injury, a few animals in the Injurious  $V_T$  group developed a pneumothorax and required euthanization prior to 6 hr. There were no differences in survival based on the surfactant additive assignments (Table 1). Only animals that completed 6 h ventilation were included in molecular analysis. There were no differences in birth weight or GAs ( $126 \pm 1$  days).

### Physiology

In protective  $V_T$  animals, the animals receiving 0.1 mg/kg had slightly higher  $V_T$  at 15 minutes but no differences in  $V_T$  occurred throughout 6 hour ventilation period (Table 1). There were no differences in the ventilator efficiency index (VEI) or the oxygen index (OI) between protective  $V_T$  with different amounts of budesonide at 6 hours. The mean airway pressure (MAP) was lower in protective  $V_T + \text{Bud } 0.25$  and protective  $V_T + \text{Bud } 0.1$  compared to Saline animals, and dynamic compliance was higher in  $+ \text{Bud } 0.25$  mg/kg animals. The Volume at 40 cmH<sub>2</sub>O ( $V_{40}$ ) at necropsy was higher in protective  $V_T + \text{Bud } 0.1$  and  $+ \text{Bud } 0.04$  mg/kg groups. Due to low compliance and surfactant deficiency in the injury  $V_T$  animals, the maximal pressure of 50 cmH<sub>2</sub>O only produced between 8 to 9 mL/kg in part because the injury  $V_T$  was delivered without PEEP (Table 1). After receiving surfactant, injury  $V_T$  animals had similar tidal volumes throughout the ventilation period using a PEEP of 5 cmH<sub>2</sub>O, with lower pressure requirements. Injury  $V_T + \text{Bud } 0.25$  had improved VEI

compared with injury  $V_T$  + Saline animals, whereas injury  $V_T$  + Bud 0.1 and +Bud 0.04 mg/kg had VEI similar to Saline only animals. The MAP was also lower in injury  $V_T$  + Bud 0.25 animals, and compliance was higher in all budesonide exposed injury  $V_T$  compared to injury  $V_T$  + Saline. V40 was also higher with Bud 0.25 mg/kg than in the injury  $V_T$  + Saline animals. Overall, injury  $V_T$  animals exposed to budesonide 0.1 mg/kg or 0.04 mg/kg did not have the same degree of beneficial effects on physiology as animals receiving Bud 0.25 mg/kg.

### Budesonide levels

Budesonide could be measured in the plasma by 15 minutes after intra-tracheal dosing in all animals receiving budesonide (Figure 1). The dose at each time point was proportionate to the initial dose given and plasma levels remained significantly different between doses across the ventilation period in both animal groups receiving protective  $V_T$  and injury  $V_T$ . The plasma level decreased over time but remained measurable at 6 hours after dosing. The mean plasma level for protective  $V_T$  + Bud 0.25 mg/kg ( $170 \pm 78$  ng/mL) was higher at 15 min than the plasma level for injury  $V_T$  0.25 mg/kg ( $104 \pm 10$ ) ( $p=0.03$ ). By 30 minutes, there were no differences in the plasma levels between the protective  $V_T$  and injury  $V_T$  groups. The amount of budesonide extracted from the lungs after 6 hours was also proportionate to the dose given (Table 2). The percent of initial dose remaining in the lung (as free drug + esters) was low, between 2.2% to 3.5%, and was similar between all groups. Lung tissue was hydrolyzed to remove budesonide esters. In the lungs treated with 0.25 mg/kg budesonide, there was about 40% of total budesonide remaining in the lung as budesonide esters. In protective  $V_T$  lambs, there were less budesonide esters formed after the 0.1 mg/kg budesonide treatment (14%) and 0.04 mg/kg treatment (19%).

### Lung and liver responses

Mechanical ventilation caused increased pro-inflammatory cytokine mRNA levels with both protective  $V_T$  and injury  $V_T$  ventilation compared to unventilated controls (fold increase set to 1) (Table 3). In protective  $V_T$  animals, mRNA for the pro-inflammatory cytokines IL-6 and MCP-1 decreased in the lung with budesonide 0.25 mg/kg compared to Saline animals. Protective  $V_T$  + Bud 0.1 and 0.04 mg/kg did not change the lung cytokine mRNA responses. There were no significant differences in lung injury scores or the number of iNOS positive inflammatory cells on lung histology between the protective  $V_T$  groups, regardless of budesonide exposures (Table 3). The mRNA for the epithelial sodium channel (ENaC) was increased in all ventilated animals compared with unventilated controls (Table 3), and further increased in protective  $V_T$  + Bud 0.25 compared with protective  $V_T$  + Saline. MCP-1 mRNA was decreased in the liver of the protective  $V_T$  + Bud 0.25 and 0.1 mg/kg animals compared to protective  $V_T$  + Saline, with no difference with protective  $V_T$  + Bud 0.04 mg/kg. There were no differences in liver Serum Amyloid A3 (SAA3) mRNA between groups.

In injury  $V_T$  + Saline animals there were large increases in mRNA for IL-1 $\beta$ , IL-6, and MCP-1 in the lung tissue and MCP-1 in the liver (Table 3). Injury  $V_T$  + Bud 0.25 mg/kg had decreased mRNA for IL-1 $\beta$ , I-6, and MCP-1 in the lung and MCP-1 mRNA in the liver compared with Saline animals. Histologic injury scores and iNOS positive cells in the lung



also decreased with Bud 0.25 mg/kg. In animals receiving injury  $V_T$ , Bud 0.1 mg/kg or 0.04 mg/kg did not statistically decrease any markers of injury in the lung or liver. With injury  $V_T$ + Bud 0.1 mg/kg, there were nearly significant changes, with non-parametric testing, for IL-1 $\beta$  ( $p=0.06$ ), MCP-1 ( $p=0.06$ ), and iNOS scoring in lung ( $p=0.06$ ). ENaC mRNA increased with injury  $V_T$  and further increased with all three doses of budesonide. SAA3 mRNA decreased in the liver with Bud 0.1 mg/kg but not in other doses compared with injury  $V_T$  + Saline.

### Discussion:

Because of concerns about the systemic release of budesonide from the lungs, lower doses were evaluated for efficacy in decreasing lung inflammation. Unfortunately, the lower doses (0.1 mg/kg and 0.04 mg/kg) were less effective in decreasing lung inflammation and other markers of injury. All three doses had some effects on the physiology, demonstrating some beneficial effect with even 0.04 mg/kg. Similar to our experiences with 0.25 mg/kg of budesonide, the budesonide was found in the plasma of both protective  $V_T$  and injury  $V_T$  animals and was proportional to the amount given at birth. The inconsistent responses at the 0.1 mg/kg is concerning for planning large multicenter trial, and suggests the 0.25 mg/kg dose used by Yeh *et al.* may be necessary to decrease the rates of BPD.

The budesonide levels in the plasma and lung correlated with the dose of budesonide given and were similar in both the Protective and Injury  $V_T$  animals. The same percent of the dose of budesonide was found in the plasma at all dose levels suggesting it is quickly absorbed from the lung. The plasma levels found at 1 hour in budesonide 0.1 mg/kg group (15 ng/ml in protective  $V_T$  and 16 ng/ml in injury  $V_T$ ) is similar to preterm infants treated with surfactant with budesonide 0.1 mg/kg (14 ng/ml) in a pilot study in preterm infants (23). In preterm infants receiving 0.05 mg/kg, the 1 hour blood level was 6 ng/mL, which is similar to the 7 ng/mL in the lambs receiving 0.04 mg/kg (23). The similarities between the human dosing, determined by MS-LC from dried blood spots, and lamb dosing at the lower doses suggests the pharmacology determined for the 0.25 mg/kg in sheep would correlate with similar human values. The budesonide plasma levels at 1 hour for budesonide 0.25 mg/kg (50 ng/mL) was higher than previously reported by Roberts *et al.* (25 ng/mL) in more mature sheep (132 d GA) who received antenatal steroids, suggesting that gestational age and antenatal steroids may influence blood levels (19). In their initial pilot study, Yeh *et al.* found 20 ng/mL at 30 min and 18 ng/mL at 1 hour after an initial 0.25 mg/kg dose given at an average if 2 hours of life. We found slightly lower plasma budesonide levels at 15 minutes in the injury  $V_T$  group compared to protective  $V_T$  group, which might suggest a role of lung fluid clearance on systemic release of budesonide. As with previous experiments, less than 4% of original dose at 6 hrs was detected in the lung tissue at all treatment doses (6). There were proportionately less budesonide esters in the lung tissue in the lower doses than 0.25 mg/kg, thus the prolonged anti-inflammatory effects found by Yeh *et al.* may not occur in the lower doses (11). We have previously demonstrated very low levels of budesonide in the bronchoalveolar lavage fluid at the end of ventilation (4) thus did not analyze the lower doses. We previously demonstrated that a higher dose of 1.0 mg/kg did not have additional benefits over 0.25 mg/kg in ventilated preterm fetal sheep (20).

The lower doses had variable effects on lung inflammation and injury responses, whereas the 0.25 mg/kg budesonide dose was more consistent. Yeh *et al.* demonstrated decreased IL-8 in the BAL at up to 8 days after the 0.25 mg/kg budesonide doses (11, 12). In pilot dosing studies by Ballard *et al.*, presented in abstract form, there were inconsistent decreases in IL-8 in BAL samples with about half the infants responding in the 0.1 mg/kg dose, as well as other doses tested (32). These inconsistent anti-inflammatory responses were also seen in these lambs, with some of the lambs demonstrating large decreases in cytokine mRNA and others with minimal change from surfactant only animals. These inconsistencies can be seen in the large standard deviations in the markers of injury. To try to explain the inconsistencies at the lower doses, we examined the plasma and lung budesonide levels in relation to other markers of injury and found no correlations between the budesonide levels in the plasma and lung and any of the physiologic or injury markers. Of interest, for almost all markers of injury in the lungs, the 0.25 mg/kg had qualitatively less injury than the 0.1 mg/kg dose which was less than the 0.04 mg/kg dose. The lowest dose was not entirely inactive, as it increased the mRNA for the epithelial sodium channel (ENaC) in the lung. The ENaC channel is important for lung fluid clearance at birth, increases near the term in infants, and can be induced by antenatal steroids (20). Systemically, the 0.25 mg/kg and 0.1 mg/kg both decreased the MCP-1 mRNA in the liver. We have previously demonstrated by mRNA sequencing multiple pathways altered in the liver and brain with mechanical ventilation and budesonide (6).

Our experiment has some limitations due to using animal group sizes of 5 to 6 per group. Some differences in the 0.1 mg/kg group were nearly significant and higher numbers may have showed increased benefit in this group. The ewes also did not receive antenatal betamethasone, and this could alter the effects. Death and gender imbalances may have affected lung physiology measurements. The two interventions were designed to be extremes of injury and protective ventilation, thus normal delivery room interventions likely are a combination of the two. We intentionally used multiple variables associated with injury at birth (33, 34) to create severe injury, and thus we are unable to determine the specific ventilation variables that were sensitive to budesonide. In previous fetal models without oxygen exposure, we have demonstrated systemic response to mechanical ventilation (35). The Protective  $V_T$  animals received surfactant prior to ventilation which does not occur clinically. Although the ventilation strategy was labelled protective, these animals still had significant pro-inflammatory responses compared with unventilated controls.

While the Yeh *et al.* studies randomized preterm infants with high oxygen ( $FiO_2 > 0.50$ ) and ventilator support requirements prior to enrolment, Kothe *et al.* treated all infants failing CPAP with budesonide 0.25 mg/kg in an observational study (11, 12, 14). These studies gave an average of two doses of the surfactant and budesonide combination. Since the majority of the budesonide has been absorbed from the lungs in the preterm sheep by 6 hours, some of the benefit from the budesonide and surfactant in these trials may be from the second dose given. None of these studies found serious systemic effects of the budesonide treatment, and long-term neurologic follow-up was not different between groups (11, 36). In studies of inhaled budesonide over weeks, BPD decreased but there was a slight increase in mortality (10). These studies, which exposed infants to much larger doses of budesonide,



also did not demonstrate other systemic effects (10). Although applying different inclusion criteria, additional clinical trials are currently underway to confirm these effects.

### Conclusions:

At birth, single lower doses of budesonide (0.1 mg/kg and 0.04 mg/kg) were not as effective as budesonide 0.25 mg/kg at decreasing lung inflammation from Protective  $V_T$  or Injurious  $V_T$ . Inconsistent responses were seen in the lambs receiving 0.1 mg/kg and even less effect was seen at the lowest dose. Although there were less effects on proinflammatory cytokines with the lower doses, many of the physiologic benefits found with the 0.25 mg/kg dose were also found with 0.1 mg/kg and some with the lowest dose. At all budesonide doses evaluated, regardless of the type of ventilation received, the majority of the budesonide was no longer in the lungs after 6 hours of ventilation. Previous experiments in fetal sheep demonstrated no additional benefit from higher dosing (budesonide 1.0 mg/kg) versus budesonide 0.25 mg/kg (20). The dose Yeh *et al.* used in their clinical studies appears to be the most effective dose tested for decreasing lung injury, though effects from the systemic release of the budesonide should be monitored in newborns.

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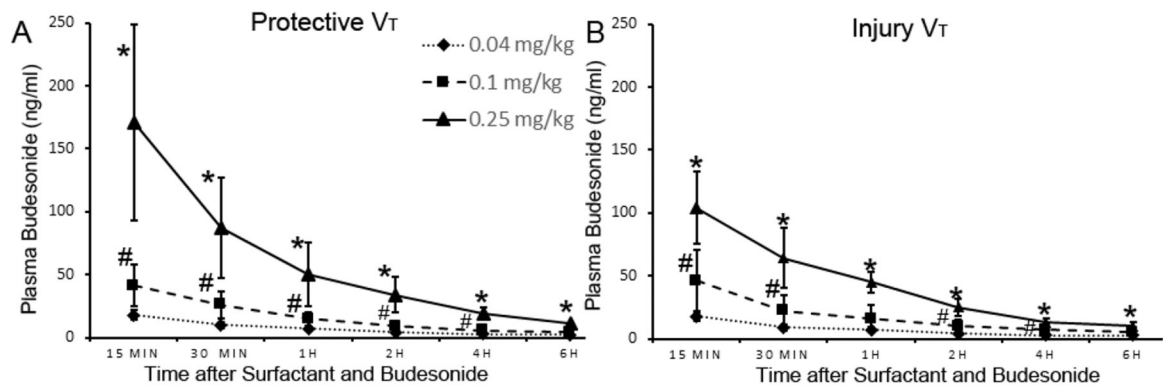
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**Impact Questions:**

1. The addition of budesonide to surfactant decreases the lung and systemic responses to both protective and injurious mechanical ventilation in preterm sheep
2. Budesonide levels in the plasma are proportionate to the dose given and the majority of the budesonide is no longer in lung at 6 hours of ventilation
3. Budesonide 0.25 mg/kg is more efficacious for decreasing cytokine levels/ inflammatory biomarkers than the lower budesonide doses of 0.10 mg/kg and 0.04 mg/kg
4. The budesonide dose of 0.25 mg/kg being used clinically and in current clinical trials seems likely to decrease lung inflammation in preterm infants



**Figure 1:**

Plasma Budesonide levels across time. (A) Plasma levels of animals receiving surfactant and budesonide prior to ventilation (protective  $V_T$ ) were proportionate to the dose given at every time point and decreased across time. A significant dose was present in the plasma by 15 minutes after intra-tracheal dose. (B) Animals receiving 15 minutes of mechanical ventilation prior to surfactant and budesonide (Injury  $V_T$ ) had a similar pattern of budesonide in the plasma. Mean  $\pm$  SEM. \*  $p < 0.05$  vs 0.1 and 0.04 mg/kg; #  $p < 0.05$  vs 0.04 mg/kg

**Table 1:**

Animals data and ventilation physiology

Surfactant Additive	Saline	Bud 0.25 mg/kg	Bud 0.1 mg/kg	Bud 0.04 mg/kg
<b>Protective V<sub>T</sub> ventilation</b>				
Number in group	6	6	6	5
Birth weight (kg)	3.1±0.4	3.1±0.3	2.8±0.3	2.9±0.4
Male:Female	3:3	3:3	1:5	1:4
V <sub>T</sub> at 15 min (ml/kg)	7.0±0.8	6.7±0.9	<b>8.6±0.5 *</b>	7.7±0.4
MAP at 15 min (cmH <sub>2</sub> O)	16.0±1.2	16.2±1.0	16.1±1.0	17.1±1.2
VT average for 6 hour	7.6±0.4	7.6±0.8	7.6±0.8	7.5±0.4
MAP average for 6 hour	14.2±1.5	13.6±0.8	13.5±1.0	13.8±1.0
VEI at 6 hour	0.07±0.03	0.10±0.02	0.09±0.01	0.08±0.01
OI at 6 hour	49±52	20±16	11±7	36±55
MAP at 6 hour (cmH <sub>2</sub> O)	14.8±2.3	<b>12.2±0.8 *</b>	<b>12.1±0.7 *</b>	13.0±1.4
Compliance (mL/cmH <sub>2</sub> O/kg)	0.30±0.08	<b>0.40±0.08 *</b>	0.37±0.10	0.40±0.05
V40/kg (mL)	27±8	36±8	<b>47±9 *</b>	<b>42±6 *</b>
<b>Injury V<sub>T</sub> for first 15 minutes</b>				
Initial Animals in Group	10	9	9	6
Surviving Animals	9	7	5	5
Birth weight (kg)	3.0±0.4	3.3±0.5	2.9±0.3	3.0±0.3
Male:Female	2:7	2:5	2:3	3:2
V <sub>T</sub> at 15 min (ml/kg)	7.8±1.4	7.9±1.3	8.9±1.4	7.9±1.2
MAP at 15 min (cmH <sub>2</sub> O)	23.8±0.0	23.6±0.5	23.8±0.1	23.8±0.0
VT average for 6 hour	7.7±0.8	8.1±0.7	8.1±0.6	7.9±0.4
MAP average for 6 hour	18.0±1.3	<b>16.1±0.7t</b>	17.1±1.0	17.1±0.7
VEI at 6 hour	0.04±0.02	<b>0.07±0.02t</b>	0.05±0.02	0.05±0.03
OI at 6 hour	63±48	53±40	82±62	74±76
MAP at 6 hour (cmH <sub>2</sub> O)	17.7±2.8	<b>14.0±1.2t</b>	15.4±1.4	16.0±1.4
Compliance (mL/cmH <sub>2</sub> O/kg)	0.23±0.06	<b>0.35±0.06t</b>	<b>0.35±0.02t</b>	<b>0.31±0.05t</b>
V40/kg (mL)	24±10	<b>36±7t</b>	34±3	28±6

Mean±SD

\* p < 0.05 vs Protective V<sub>T</sub> + Saline; t p < 0.05 vs Injury V<sub>T</sub> + Saline V<sub>T</sub>= Tidal Volume, MAP=Mean Airway Pressure, VEI=Ventilation efficiency index, OI=Oxygenation index



**Table 2:**

Budesonide levels in lung tissue at 6 hr.

Group	Initial Dose	Protective V <sub>T</sub> Animals			Injury V <sub>T</sub> Animals		
		Free Budesonide	Budesonide Esters	% of initial dose †	Budesonide	Budesonide Esters	% of initial dose †
<b>0.25 mg/kg</b>	750 µg	15.5±4.1µg	7.8±1.3µg	3.1±0.6%	19.1±7.6µg	7.4±14.1µg	3.5±1.7%
<b>0.10 mg/kg</b>	300 µg	5.9±1.6µg	0.7±1.6µg	2.2±0.6%	6.5±3.0µg	1.4±5.8µg	2.4±1.3%
<b>0.04 mg/kg</b>	120 µg	2.7±1.6µg	0.3±0.4µg	2.5±1.3%	3.4±0.4µg	2.7±0.µg	2.9±0.6%

Mean±SD n= 5 to 8 animals per group

†(Free budesonide + Budesonide esters)/Initial dose given\*100=% of initial dose at 6 hr.

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**Table 3:**

Markers of injury in the lung and liver

Surfactant Additive	Controls	Saline	Bud 0.25	Bud 0.10	Bud 0.04
<b>Protective V<sub>T</sub> ventilation (n=5-6/group)</b>					
Lung IL-1 $\beta$ mRNA	1 $\pm$ 0.6	11 $\pm$ 7	6 $\pm$ 7	9 $\pm$ 5	21 $\pm$ 20
Lung IL-6 mRNA	1 $\pm$ 0.6	75 $\pm$ 26	<b>22<math>\pm</math>18</b> *	55 $\pm$ 55	151 $\pm$ 144
Lung MCP-1 mRNA	1 $\pm$ 0.3	99 $\pm$ 93	<b>15<math>\pm</math>13</b> *	31 $\pm$ 33	74 $\pm$ 67
Lung ENAC mRNA	1 $\pm$ 0.1	20 $\pm$ 11	<b>66<math>\pm</math>24</b> *	45 $\pm$ 20	42 $\pm$ 30
Lung Injury Score (out of 8)	0.4 $\pm$ 0.3	4.5 $\pm$ 2.5	3.7 $\pm$ 2.0	2.0 $\pm$ 0.7	3.0 $\pm$ 1.7
Lung iNOS+ cells (out of 2)	0 $\pm$ 0	0.7 $\pm$ 0.8	0.3 $\pm$ 0.8	0.1 $\pm$ 0.2	0.3 $\pm$ 0.4
Liver MCP-1 mRNA	1 $\pm$ 0.3	7 $\pm$ 7	<b>0.5<math>\pm</math>0.4</b> *	<b>0.6<math>\pm</math>0.6</b> *	3 $\pm$ 3
Liver SAA3 mRNA	1 $\pm$ 1	20 $\pm$ 7	52 $\pm$ 52	22 $\pm$ 19	29 $\pm$ 18
<b>Injurious Ventilation for first 15 minutes (n=5-8/group)</b>					
Lung IL-1 $\beta$ mRNA	1 $\pm$ 0.6	104 $\pm$ 112	<b>10<math>\pm</math>15t</b>	16 $\pm$ 29	11 $\pm$ 11
Lung IL-6 mRNA	1 $\pm$ 0.6	2117 $\pm$ 2876	<b>59<math>\pm</math>89t</b>	87 $\pm$ 75	183 $\pm$ 182
Lung MCP-1 mRNA	1 $\pm$ 0.3	376 $\pm$ 347	<b>39<math>\pm</math>23t</b>	74 $\pm$ 60	72 $\pm$ 57
Lung ENAC mRNA	1 $\pm$ 0.1	20 $\pm$ 9	<b>37<math>\pm</math>19t</b>	<b>34<math>\pm</math>11t</b>	<b>41<math>\pm</math>20t</b>
Lung Injury Score (out of 8)	0.4 $\pm$ 0.3	6.3 $\pm$ 1.6	<b>4.4<math>\pm</math>1.5t</b>	5.0 $\pm$ 1.1	5.9 $\pm$ 2.6
Lung iNOS+ cells (out of 2)	0 $\pm$ 0	1.2 $\pm$ 0.7	<b>0.5<math>\pm</math>0.6t</b>	0.5 $\pm$ 0.5	0.9 $\pm$ 1.2
Liver MCP-1 mRNA	1 $\pm$ 0.3	79 $\pm$ 103	<b>9<math>\pm</math>21t</b>	11 $\pm$ 11	7 $\pm$ 7
Liver SAA3 mRNA	1 $\pm$ 1	79 $\pm$ 103	66 $\pm$ 43	<b>26<math>\pm</math>12t</b>	37 $\pm$ 30

mRNA data represents fold change compared to unventilated controls. All surviving animals to 6 hours were analyzed. Mean $\pm$ SD

\* p < 0.05 vs Protective V<sub>T</sub> + Saline, t = p < 0.05 vs Injury V<sub>T</sub> + Saline