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Maternal and Intra-amniotic Corticosteroid Effects on Lung Morphometry in Preterm Lambs

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ABSTRACT: We investigated the effects of intra-amniotic (IA) betamethasone or budesonide injections on lung structure 2 or 7 d after treatment in preterm fetal sheep. Pregnant ewes received intra-amniotic betamethasone (2 mg/kg fetal weight), budesonide (2 mg/kg), saline or maternal intramuscular betamethasone (0.5 mg/kg maternal weight), or saline. Lambs were delivered 2 or 7 d later at 124 d of gestation. Morphometric analysis was conducted on the right upper lung lobe. Intra-amniotic corticosteroids, 2 or 7 d before delivery, resulted in higher average alveolar volumes than controls (2 d: 25%–35%, 7d: 15%–25%). All corticosteroid treatments resulted in thinning of alveolar walls 7 d after treatment and a higher proportion of alveolar ducts and a lower alveolar wall fraction relative to controls 2 or 7 d after treatment. The changes in structural lung indices correlated with the improved lung function at 2 d. Structural lung indices and increased surfactant correlated with the improved lung function at 7 d. Similar structural changes induced by intra-amniotic corticosteroids and maternal intramuscular betamethasone were associated with improvements in lung function at 2 and 7 d. **(*Pediatr Res* 62: 32–36, 2007)**

Antenatal corticosteroids are used routinely to induce fetal lung maturation and prevent respiratory distress syndrome (RDS) in preterm infants (1,2). The widespread adoption of antenatal corticosteroid therapy has improved outcomes for thousands of preterm babies (2), but the benefits are not consistent. Antenatal corticosteroids reduce the risk of RDS by approximately 50%, but the protection against the development of bronchopulmonary dysplasia (BPD) (3,4) does not occur after a single course of treatment, although repetitive treatment causes may be protective (5). The possibility of augmenting the maturational effects of antenatal corticosteroids has been the subject of many studies, but, to date, there is no other proven effective therapy (6).

Our observations that IA endotoxin induces preterm lung maturation by direct contact of endotoxin with the fetal lung (7,8) led us to investigate the maturational potential of IA corticosteroids. IA administration would reduce the risks to the fetus associated with direct fetal i.m. injection and minimize the unwanted and potentially harmful effects of systemic maternal administration, particularly for women with diabetes mellitus. Although IA corticosteroids were shown to mature the fetal lungs (9,10), we wanted to verify that the changes to lung maturation were similar to those previously described by other routes. Due to the possible systemic take up of corticosteroids by the fetus (10,11), we also investigated the maturational effects of budesonide, a corticosteroid preparation designed to be administered by inhalation in postnatal life, that has low systemic bioavailability (6%) due to rapid effective clearance from plasma (12,13).

IA betamethasone or budesonide (0.5 or 2 mg/kg estimated fetal body weight) improved preterm lung function 2 d after treatment, equivalent to that induced by maternal betamethasone (14). These improvements in function were not accompanied by increases in pulmonary surfactant. Lung maturation induced by IA betamethasone or budesonide also was associated with high rates of injury when the preterm lung was ventilated (14). Therefore, we hypothesized that improved respiratory function after IA exposure to corticosteroids was due to structural alterations within the lung. We compared the effects of IA corticosteroids and maternal i.m. betamethasone and related these structural effects to previously reported indices of preterm lung function in these same animals (14).

METHODS

All experimental procedures were approved by the animal ethics committees from the Cincinnati Children's Hospital Medical Centre and the Department of Agriculture and Food, Western Australia. Pregnant ewes bearing single fetuses were treated with i.m. medroxyprogesterone acetate (150 mg; Depo-Ralovera, Kenral, Australia) to prevent glucocorticoid-induced preterm labor (15). Maternal i.m. betamethasone (0.5 mg/kg maternal body weight; Celestone Chronodose, Schering Plough, Baulkham Hills, NSW, Australia), and IA injections (total volume of 2 mL) of either saline solution, betamethasone (0.5 or 2.0 mg/kg estimated fetal body weight) or budesonide (0.5 or 2.0 mg/kg estimated fetal body weight; Pulmicort Respules, AstraZeneca, North Ryde, NSW, Australia) were given according to an established protocol (16). Preterm lambs were delivered by cesarean section at 123–125 d of gestation (both 2- and 7-d groups), dried, and ventilated for 40 min for assessment of lung function (Fio_2: 100%, 40 breaths per minute, 3 cm H_2O positive end-expiratory pressure with peak inspiratory pressure adjusted to achieve a target $Paco_2$ of 50 mm Hg). Visual examination of the lungs at autopsy determined the existence of pulmonary interstitial emphysema (PIE). Functional outcomes have been reported previously (14).

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Abbreviations: BPD, bronchopulmonary dysplasia; **IA,** intra-amniotic; **PIE,** pulmonary interstitial emphysema; **RDS,** respiratory distress syndrome; **VEI,** ventilator efficiency index

Tissue collection and preparation. The right upper lobe (RUL) was inflation fixed at 30 cm H_2O distending pressure overnight *via* bronchial instillation of 10% formalin. Fixed lobe volume (FLV) was measured by the Scherle volume displacement method (17). Each lobe was cut into 5-mm serial slices, and three slices were randomly selected and uniformly embedded in paraffin (18). The paraffin embedded sections were then cut into 5-μm sections and subsequently stained with hematoxylin and eosin (H&E) for morphometric measurements. All morphometric assessments were performed on the RUL to account for regional maturation variability known to exist within the lungs (19,20) and were performed blind by a single operator (G.R.P.).

Morphometry. Stained sections of lung were digitally photographed and digitally enlarged, and a cycloid point counting grid (72 lines/144 points) was superimposed onto each photographic image to calculate the volume fraction of lung parenchyma (PF; alveoli and alveolar ducts), nonparenchyma (NPF; blood vessels and conducting airways), interlobular septa (ISF; interstitial tissue forming distinct lobulation of the lung), and pleura (PLF), as described previously (7). Volume fraction is equal to the number of test points on the structure of interest, PI (*e.g.* pleura), divided by the total number of points for all compartments, Pt. Total volumes of parenchyma and other compartments were derived from volume fraction multiplied by FLV.

Digitized images from 10 nonoverlapping parenchymal fields were captured from each 5-μm section and examined at a magnification of $\times$950. The number of points that fell on air space and on alveolar septal tissue and the number of air/tissue tissue/air intercepts were counted by digital superimposition of a linear point counting grid (36 lines/72 points). The surface area of alveoli and alveolar ducts per unit volume of parenchyma (S_v) was calculated using the formula $S_v = 2I_o/I_r$, were I_o is the number of intercepts with the air/tissue interface and L_r is the length of the test line within the reference volume. Total alveolar surface area is $S_v \times FLV \times PF$, where FLV is the fixed lung volume. Alveolar wall thickness was determined as volume per unit area of alveolar surface using the formula AWF/S_v, where AWF is the volume fraction of alveolar wall tissue. Alveolar numerical density (number per unit volume) was calculated according to the method of Weibel (21) using the equation $N_v = N_A^{3/2}/(B \times AF^{1/2}) \times D$.

where N_A = number of alveoli per unit area, AF = volume fraction of alveolar airspace, B = shape constant describing alveolar shape (=1.55), and D = distribution variable of the characteristic linear dimension of the alveoli (taken to equal 1). In transverse section, alveoli were identified as structures opening onto a common air space (alveolar duct). In cross section, alveoli were defined as those structures wholly enclosed by respiratory epithelium. Relative size and the presence of secondary alveolar septa distinguished alveolar ducts from alveoli. Size and morphology (wall thickness and cellularity) were used to distinguish alveoli from saccules. Ambiguous structures were rejected. The total number of alveoli in the right upper lobe was calculated by multiplying lobe volume by N_v. Average alveolar volume (V_A) was calculated by dividing total alveolar volume (FLV $\times$ PF $\times$ AF) by total alveolar number.

Statistical analysis. Preliminary analyses indicated that data from IA saline groups given 2 or 7 d before delivery were similar, so data from these treatment groups were combined to form a single control group. Comparisons between groups at either 2 or 7 d after treatments were made using analysis of variance (ANOVA). Comparisons of normally distributed corticosteroid groups *versus* controls at 2 or 7 d after treatment were conducted using a two-way ANOVA. Nonparametric data were evaluated using the Mann-Whitney *U* test. Lung function indices of ventilation efficiency index (22) (VEI; an index that integrates ventilation with respiratory support), compliance, and lung volume at 40 cm H_2O pressure (as determined by deflation pressure volume curves) reported previously (14) were used for multivariant analyses using a generalized linear model (SPSS v12.0.1), to determine which structural variables were predictors of lung function. Data are presented as mean $\pm$ SEM. Statistical significance was accepted at $p < 0.05$.

RESULTS

Fetal outcome. All fetuses were healthy, as indicated by their cord arterial blood gas and acid/base status immediately at the time of delivery as reported previously (14). Body weights of lambs were not different between groups (14). We previously reported a high rate of pregnancy loss (50%) in ewes treated with 2.0 mg/kg IA betamethasone scheduled for delivery 7 d after treatment (14).

Lung morphometry. FLV and the volume fraction of lung parenchyma were similar 2 or 7 d after saline or corticosteroid treatment (Tables 1 and 2). The volume of interstitial tissue was lower than for control lambs delivered 7 d after corticosteroid treatment: interlobular septal volume decreased by 27%–50% in corticosteroid treated animals ($p < 0.005$ for all). Interlobular septal volume was lower than in controls for lambs delivered 7 d after IA budesonide or i.m. betamethasone ($p < 0.05$), but the parenchymal volume fraction was significantly increased 7d after IA budesonide or IM betamethasone treatment ($p < 0.05$; Table 2). These results reflect a higher volume of air space and a lower interstitial tissue volume, indicating a larger gas volume in the lungs after corticosteroid exposure.

The alveolar wall volume fraction was 5% to 7% lower than control 2 d after all corticosteroid treatments and was further reduced (8%–11%) 7 d after treatment ($p < 0.005$ for all treatment groups at both time points *versus* control). Alveolar wall thickness was lower than control 7 d after exposure to any corticosteroid treatment ($p < 0.001$; Table 2). These results indicate that IM or IA corticosteroids, 2 or 7 d before delivery, resulted in alveoli with thinner walls than those of the control.

The alveolar air space volume fraction was not affected by corticosteroid treatment. Alveolar surface area per unit vol-

Table 1. *Lung morphometry and function 2 d after treatment*

2-d treatment groups	Control (n = 6)	Mat beta 0.5 mg/kg (n = 7)	IA beta 2.0 mg/kg (n = 7)	IA bud 0.5 mg/kg (n= 5)	IA bud 2.0 mg/kg (n = 8)
Compliance (mL/kg/cm H_2O)	0.18 ± 0.01	0.30 ± 0.02	0.34 ± 0.03*	0.30 ± 0.02*	0.34 ± 0.03*
VEI	0.03 ± 0.00	0.05 ± 0.00*	0.06 ± 0.01*	0.05 ± 0.01*	0.05 ± 0.01*
Saturated phosphatidylcholine(μmol/kg)	0.15 ± 0.02	0.20 ± 0.04	0.20 ± 0.06	0.36 ± 0.14*	0.11 ± 0.07
Fixed lobe volume (mL)	16.6 ± 0.8	18.3 ± 1.4	19.4 ± 1.7	19.6 ± 1.7	20.0 ± 1.4
Parenchymal volume fraction	0.74 ± 0.01	0.73 ± 0.02	0.74 ± 0.02	0.75 ± 0.03	0.78 ± 0.02*
Interlobular septal volume (mL)	2.2 ± 0.1	2.4 ± 0.3	1.9 ± 0.2	2.8 ± 0.5	2.0 ± 0.1
Alveolar wall volume fraction (%)	43.8 ± 1.0	37.3 ± 1.3*	38.1 ± 1.1*	40.1 ± 2.8	38.0 ± 1.0*
Alveolar air space volume fraction (%)	8.0 ± 0.7	5.5 ± 0.4	6.4 ± 0.6	3.7 ± 0.4*	4.9 ± 0.4*
Alveolar duct volume fraction (%)	48.2 ± 1.3	57.2 ± 1.5*	55.9 ± 1.6*	56.1 ± 2.8*	58.9 ± 1.4*
Air space volume fraction (%)	56.2 ± 1.0	62.7 ± 1.3*	62.0 ± 1.3*	59.9 ± 2.8*	64.0 ± 1.1*
Alveolar wall thickness (μm)	5.2 ± 0.2	4.2 ± 0.1	4.5 ± 0.2	4.8 ± 0.2	4.5 ± 0.2
Surface fraction (mm^2/mm^3)	85.8 ± 2.4	88.3 ± 2.5	83.3 ± 3.1	82.8 ± 4.0	80.9 ± 3.2
Total surface area (mm^2)	1047 ± 48	1193 ± 93	1150 ± 125	1216 ± 124	1273 ± 122

Mat Beta, maternal betamethasone injection; IA Beta, IA betamethasone; IA Bud, IA budesonide.
* $p < 0.05$.

Table 2. *Lung morphometry and function 7 d after treatment*

7-d treatment groups	Control ($n = 6$)	Mat beta 0.5 mg/kg ($n = 6$)	IA beta 2.0 mg/kg ($n = 5$)	IA bud 2.0 mg/kg ($n = 4$)
Compliance(mL/kg/cm H_2O)	0.18 ± 0.01	0.34 ± 0.05*	0.22 ± 0.03	0.31 ± 0.04
VEI	0.03 ± 0.00	0.06 ± 0.01*	0.04 ± 0.09	0.05 ± 0.01*
Saturated phosphatidylcholine(μmol/kg)	0.15 ± 0.02	1.77 ± 0.53*	0.52 ± 0.19*	1.34 ± 0.26*
Fixed lobe volume (mL)	16.6 ± 0.8	17.3 ± 2.5	16.4 ± 0.8	18.7 ± 1.3
Parenchymal volume fraction	0.74 ± 0.01	0.81 ± 0.02*	0.79 ± 0.01	0.82 ± 0.01*
Interlobular septal volume (mL)	2.2 ± 0.1	1.2 ± 0.2*	1.6 ± 0.2	1.3 ± 0.2*
Alveolar wall volume fraction (%)	43.8 ± 1.0	37.1 ± 1.8*	37.7 ± 1.0*	34.5 ± 1.3*
Alveolar air space volume fraction (%)	8.0 ± 0.7	9.4 ± 0.9	8.6 ± 0.4	11.1 ± 0.9
Alveolar duct volume fraction (%)	48.2 ± 1.3	53.5 ± 2.3*	53.7 ± 1.2*	54.4 ± 2.1*
Air space volume fraction (%)	56.2 ± 1.0	62.9 ± 1.8*	62.3 ± 1.0*	65.5 ± 1.3*
Alveolar wall thickness (μm)	5.2 ± 0.2	4.2 ± 0.2†	4.4 ± 0.2†	4.2 ± 0.1†
Surface fraction (mm^2/mm^3)	85.8 ± 2.4	89.3 ± 2.7	86.6 ± 3.1	81.5 ± 2.4
Total surface area (mm^2)	1047 ± 48	1251 ± 184	1114 ± 50	1248 ± 83″

* $p < 0.05$; † $p < 0.001$.

ume (surface fraction) and total alveolar surface area of the RUL was not different between treatment and control groups. The proportion of structures classified as alveolar ducts was 5%–7% higher than control 2 d and 8%–9% higher than control 7 d after IA or i.m. corticosteroid administration ($p < 0.05$; Tables 1 and 2). These results reflect a more ductal lung parenchyma and potentially an arrest of alveolarization.

Average alveolar volume was 35% greater than that of the control ($p < 0.001$; Fig. 3) 7 d after IA budesonide and tended to be higher 7 d after maternal IM betamethasone ($p = 0.087$; Fig. 3). As a result of the increase in alveolar volume, alveolar numerical density (number per unit volume) was 20%–30% lower in all corticosteroid-exposed animals after 2 d ($p < 0.05$), and 15%–25% lower after 7 d; only IA budesonide administration resulted in significantly lower alveolar numerical density at 7 d ($p < 0.05$; Fig. 3). Total alveolar number tended to be lower than control 2 or 7 d after corticosteroid treatment (Fig. 3).

Two days after corticosteroid exposure, volume fraction of lung parenchyma (alveoli and alveoli ducts) increased with increasing lung compliance ($p < 0.029$) and lung volume at 40 cm H_2O pressure ($p = 0.003$), *i.e.* increasing volume fraction of lung parenchyma increased compliance and the lung volume at 40 cm H_2O pressure. Alveolar numerical density was a significant structural factor predictive of VEI measurement in the multivariable model ($p = 0.002$), 2 d after corticosteroid exposure. Seven days after corticosteroid exposure, increased saturated phosphatidylcholine content and reduced alveolar numerical density were significantly associated with increased compliance ($p = 0.007$ and $p < 0.001$, respectively) and VEI ($p = 0.030$ and $p = 0.002$, respectively), whereas reduced alveolar numerical density was also a significant predictor of increased lung volume at 40 cm H_2O pressure ($p < 0.001$), 7 d after corticosteroid exposure.

DISCUSSION

We compared changes in lung structure 2 or 7 d after IA exposure to betamethasone or budesonide with saline-treated animals, and the formulation of maternal betamethasone used clinically. All corticosteroid treatments induced significant and similar structural changes in the lung parenchyma and in other compartments (Fig. 1). The lungs were characterized by fewer, larger alveoli per unit area, with thinner walls. The alterations to lung structure induced by IA corticosteroids were similar to those induced by i.m. betamethasone. However, the number of fetal deaths (Table 3), increased incidence of pulmonary interstitial emphysema, and preterm labor (14), particularly after IA betamethasone exposure in sheep, may limit the potential of IA treatment of corticosteroids for preterm lung maturation in humans.

The most striking effect of corticosteroid treatment was the reduction in alveolar numerical density after 2 d, which tended to persist, but was not significantly reduced, at 7 d. Parenchymal volume fraction was found to be a "predictor" of compliance and lung volume at 40 cm H_2O distending pressure, whereas alveolar numerical density was a predictor of VEI 2 d after corticosteroids. The structural changes of parenchymal volume fraction and alveolar numerical density are consistent with improved function in the absence of increased surfactant at 2 d (14). The reduction in alveolar numerical density suggests fewer and larger alveoli occupy the parenchyma of corticosteroid-exposed lungs, and the increase in parenchymal volume fraction is indicative of an increase in overall area for gas exchange. The air spaces are ductal in nature as evident by the higher alveolar duct volume fraction in corticosteroid-treated lungs (Fig. 2). The associated interstitial tissue thinning, lower alveolar wall volume fraction, and the lower estimated alveolar wall thickness provide the anatomic basis for the improved lung function 2 d after corticosteroid treatment (7). Conversely, saturated phosphatidylcholine content of alveolar lavage samples and alveolar numerical density were determined to be predictors of lung function (compliance, VEI, and lung volume at 40 cm H_2O distending pressure) 7 d after corticosteroids. The influence of corticosteroids on alveolar numerical density after 7 d was not as striking as that which occurred after 2 d. However, the magnitude of the increase in ductal volume fraction and air space volume fraction induced by corticosteroids at 2 and 7 d are similar, as are the physiologic changes (VEI, compliance) between the two time points. The similarities induced by corticosteroids at the different time points indicate that the improved physiology from corticosteroids is related to the structural changes in the

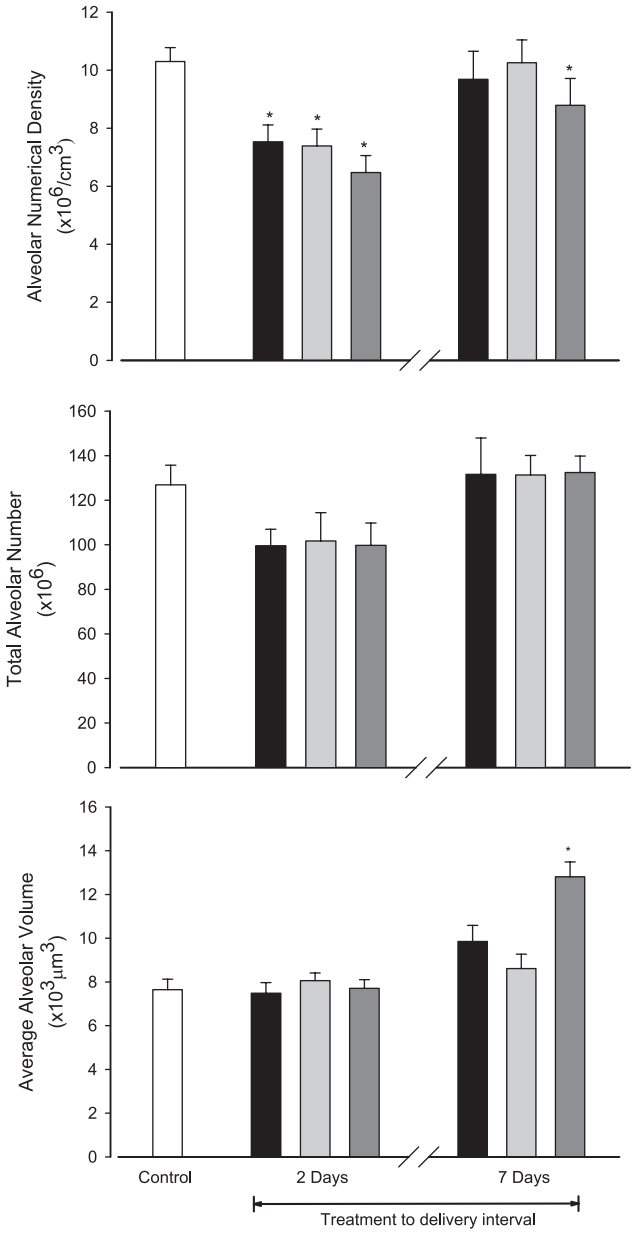

Figure 1. (*Top*) Alveolar number per unit volume of parenchymal tissue. (*Middle*) Average alveolar volume. (*Bottom*) Total alveolar number in the right upper lobe in control (□) and 2 or 7 d after treatment with i.m. betamethasone (■), IA betamethasone (▨) or IA budesonide (▩). *Significant difference from control ($p < 0.05$).

Table 3. *Incidence of pulmonary interstitial emphysema in preterm lambs exposed to corticosteroids (maternal intramuscular and intra-amniotic injection)*

Treatment to delivery interval (d)	Pulmonary interstitial emphysema
2	11/40
7	1/18

lung and confirms previous observations that improved lung function 2 d after corticosteroid treatment is due primarily to lung remodeling and not increased surfactant production (23). Our observations support the National Institutes of Health consensus of a probable benefit of corticosteroids for treatment at delivery intervals of <24 h. Further, the surfactant

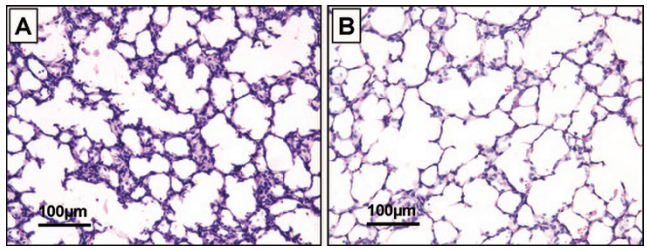

Figure 2. Light micrograph of H&E-stained 5-μm sections of lung parenchyma, from the RUL, taken at 200× magnification from a control (*A*) and a lamb exposed to IA budesonide (2.0 mg/kg fetal weight) (*B*) 7 d after their respective treatments. Note the apparent reduced wall thickness and the ductal appearance (reduced septation) of the IA lung, synergistic to lungs of BPD infants.

deficiency component of RDS is unlikely to be corrected by antenatal corticosteroids unless delivery is delayed >48 h and perhaps as long as 4–7 d.

The benefit of corticosteroids >48 h after treatment is likely augmented by increases in surfactant (24), particularly to protect against lung damage induced by postnatal ventilation (Table 3). There were high incidences (25%) of PIE and lung rupture in preterm lambs 2 d after maternal or IA corticosteroid exposure (14,25). There was only one lamb with PIE delivered 7 d after corticosteroid exposure. PIE has been associated with abnormal lung structure with alterations in pulmonary collagen and elastin content (26), and its incidence is significantly reduced by giving surfactant at birth in preterm infants who received glucocorticoids (27). Our results and those of other studies suggest that surfactant treatment at birth may protect the lungs from steroid-modulated structural alterations within the first 72 h after delivery, until surfactant production induced by corticosteroids is increased. Although the incidence of PIE in humans has not been reported in relation to time of birth after corticosteroid treatment, our results suggest that delivery <48 h after corticosteroid treatment without surfactant treatment may predispose some infants to lung injury.

At 117–122 d gestation, alveolarization is well under way in the fetal sheep lung. Although alveolar ducts normally represent the largest proportion of parenchyma at this stage of lung development, secondary septal crests, which form shallow rudimentary alveolar, are present (7,28). The alveolar stage of development is marked by the formation of millions of alveoli, thinning of the mesenchymal tissue, and a large increase in the surface area-to-volume ratio of the lung (28–30). Our results suggest a cessation of alveolarization after IA or i.m. corticosteroid treatment, consistent with previous studies (7,31–33). The corticosteroid effect is believed to be due to the inability of alveolar epithelial cells to proliferate and form secondary alveolar septa (34).

IA administration of corticosteroids may result in fetal systemic exposure through intramembranous absorption (10,11), although our experience has shown that IA agents can act locally on the fetal lung, with minimal (if any) systemic uptake (35). IA budesonide, a corticosteroid with minimal systemic availability that is commonly administered (*via* oral inhalation) in infants and young children with moderate to

severe asthma, caused structural lung maturation comparable with maternal betamethasone treatment and similar to IA betamethasone treatment after 2 or 7 d. Meta-analysis of clinical trials of early administration of inhaled corticosteroids in preterm infants found no evidence that early inhaled steroids confer important advantages over systemic steroids in the management of ventilator-dependent preterm infants (36) or reduce the incidence of BPD. However, the studies presented in the *Cochrane Review* did not start steroid treatment until 2 wk after birth, and antenatal application was not assessed. Although the effects of prolonged or repeated systemic exposure of corticosteroids on the fetus are contentious (5,37–39), this study did not assess betamethasone or budesonide concentrations in the fetal circulation or the effects of these corticosteroids on other developing organ systems.

We demonstrate that IA administration of betamethasone or budesonide did not confer any additional pulmonary structural maturational benefits beyond that achieved with maternal administration. The improved lung function without increased surfactant production found after 2 d was due to structural changes to the lung. Structural indices of alveolar numerical density and parenchymal volume fraction were predictive of postnatal lung function after 2 d, whereas alveolar numerical density and saturated phosphatidylcholine content in alveolar lavage samples were predictive of lung function 7 d after corticosteroids. Although these structural changes conferred functional improvements in the lung, the arrest in alveolarization and with fewer, larger alveoli is similar to the abnormalities that were in with infants who died of BPD associated with premature birth.

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