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Ureaplasma colonization of amniotic fluid and efficacy of antenatal corticosteroids for preterm lung maturation in sheep

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Abstract

Objective—To assess the efficacy of maternal betamethasone for improving preterm lung function, in the presence of inflammation induced by amniotic fluid ureaplasma colonization.

Study design—Ewes bearing single fetuses were randomized to receive an intra-amniotic injection of *Ureaplasma parvum* (serovar 6; 2×10^7 colony forming units) or vehicle at 86 ± 2 days of pregnancy (mean $\pm$ SD; term is 150d), followed by maternal intramuscular betamethasone (0.5mg/kg) or saline, either 2 or 7 days before delivery of lambs at 123 ± 1 d.

Results—Amniotic fluid IL-8 was elevated by ureaplasmas ($p=0.049$) but unaffected by betamethasone. Lung inflammation induced by ureaplasmas was not affected by betamethasone. Lung compliance was increased by ureaplasma colonization ($p=0.009$) and betamethasone ($p=0.042$), and effects were additive. Lung surfactant was increased by ureaplasma colonization ($p<0.001$) and betamethasone 7 days ($p=0.001$), but not 2 days, before delivery.

Conclusion—Inflammation improves preterm lung function due to increases in surfactant. Antenatal corticosteroids further augment lung function, through an apparently independent mechanism.

Keywords

betamethasone; intrauterine inflammation; infection; preterm birth

Introduction

Intrauterine inflammation, identified as clinical or histologic chorioamnionitis, is associated with preterm labour and delivery, and increased risk of neonatal morbidity and mortality ¹,

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². Ureaplasmas are common colonizers of the male and female lower genitourinary tracts ² and are the micro-organisms most-frequently associated with preterm birth ^{3, 4}. Routine amniocentesis of asymptomatic pregnancies at 15–20 weeks of gestation showed ureaplasma colonization in about 12% ^{5, 6}. Preterm prelabor rupture of membranes (PPROM) or preterm birth were more frequent when ureaplasma colonisation was present but the majority of these pregnancies continued until term ^{5, 6}; however, a diagnosis of preterm labour was made in 58% of ureaplasma-positive pregnancies in one of these studies ⁵. In another report ³, ureaplasmas were cultured from amniotic fluid and/or amnion/placental samples in 44% of pregnancies requiring Caesarean section at 23–34 weeks for PPRM or preterm labour. In a further study, *Ureaplasma* species were cultured in cord blood samples from 17% of preterm deliveries between 22–32 weeks ⁷.

Antenatal corticosteroid therapy is used universally in the management of preterm labour, principally for the prevention of respiratory distress syndrome (RDS) in neonates⁸. The presence of PPRM is not a contraindication to antenatal corticosteroid use^{9, 10}, and in many of these cases there is likely to be underlying or undiagnosed chorioamnionitis; thus, exposure to antenatal corticosteroids often occurs in the presence of ureaplasma colonisation¹¹. There are concerns that the use of corticosteroids in the presence of infection/inflammation may cause deterioration in maternal-fetal wellbeing but data relating specifically to this possibility are scarce. Antenatal corticosteroids appear not to worsen neonatal outcomes if intrauterine inflammation is present, but significant decreases in the risk of neonatal respiratory disease may not occur¹¹.

Intrauterine inflammation and ureaplasma colonization can themselves influence the likelihood of neonatal lung disease; risk of RDS is decreased^{12–14}, but risk of bronchopulmonary dysplasia (BPD) is either not affected or increased^{2, 14}. We have explored this concept, in sheep, by investigating the effects of intra-amniotic injection of lipopolysaccharide (LPS) ^{15–18} or *U. urealyticum*^{19, 20} on fetal lung development. The resultant intrauterine inflammation increases surfactant production and can induce changes in lung structure to improve preterm lung function, consistent with a reduced susceptibility to RDS. Intra-amniotic LPS injection also results in lung structural changes and evidence of abnormal surfactant secretion, which are characteristics of BPD^{18, 21}. The improvement in preterm lung function induced by intra-amniotic LPS injection can be augmented by simultaneous maternal intramuscular injection of betamethasone²²; however, the interaction between inflammation and corticosteroids is time-dependent, with an initial corticosteroid-induced attenuation of inflammation followed by augmented pro-inflammatory function^{23, 24}. Experimental assessment of the effectiveness of antenatal corticosteroids in the presence of established, chronic intrauterine inflammation has not been reported. We hypothesized that maternal intramuscular betamethasone would augment preterm lung function in lambs exposed to pre-existing amniotic fluid ureaplasma colonization.

Methods

Animal experimentation was performed in Western Australia, with approval from the Animal Ethics Committees of the Western Australian Department of Agriculture and Cincinnati Children's Hospital.

Experimental groups

Ewes bearing singleton fetuses were randomized to one of 2 groups at 86 ± 2 (mean $\pm$ SD) days of pregnancy (d) for ultrasound-guided injection of ureaplasmas (2×10^7 colony forming units; cfu) or the suspending medium alone (vehicle), as described previously²⁰. The *Ureaplasma parvum* (serovar 6) isolate was obtained from a semen sample collected at the Wesley IVF Service (Brisbane, Australia).

Medroxyprogesterone acetate (150 mg) was injected intramuscularly to all ewes at 109 ± 2 d (mean $\pm$ SD) as prophylaxis against subsequent preterm labor, which may be induced by corticosteroids in sheep²⁵. Ureaplasma and control groups were further randomised to receive a maternal intramuscular injection of betamethasone (0.5mg/kg; Celestone Chronodose, Schering Plough, Australia) or saline, either 2 days or 7 days before Caesarean delivery of preterm lambs at 123 ± 1 d (mean $\pm$ SD). This betamethasone dose reliably induces fetal lung maturation in sheep²⁵. The numbers of lambs studied in each group are presented in Table 1.

Delivery and Tissue Sampling

Ewes were killed by penetrating captive bolt, and an abdominal incision was made to locate the fetal head within the uterus. The uterus was incised without cutting the fetal membranes. A sample of amniotic fluid was collected, for subsequent culture of ureaplasmas, using a sterile needle and syringe. The fetus was delivered, the umbilical cord clamped, and pentobarbital (100 mg/kg) was injected into an umbilical vein. The cord was cut and the fetus was weighed; umbilical arterial blood was collected for blood gas and pH measurement and for total and differential white blood cell counts.

The chest of each lamb was opened, and a deflation pressure-volume curve was measured after air inflation of the lungs to a pressure of 40 cmH₂O¹⁵. The lungs were removed, each lung was weighed, and the left lung was lavaged 3 times by infusion and withdrawal of a sufficient volume of 4°C physiologic saline solution to fully fill the lung. The recovered bronchoalveolar lavage fluid (BALF) was used for cell counts¹⁷ and aliquots were collected for subsequent analysis. Pieces of the lower lobe of the right lung were rapidly frozen in liquid nitrogen and stored (−80°C) for subsequent analysis.

Biochemical analyses

Saturated phosphatidylcholine (Sat-PC) was quantified by phosphorus assay after isolation from chloroform-methanol (2:1) extracts of BALF using neutral alumina column chromatography, and exposure of lipid extracts to osmium tetroxide¹⁵. Total RNA was extracted from frozen lung tissue and chorioamnion using a modified Chomzynski method; 10 µg of total RNA was used for IL-1β, IL-6, and IL-8 mRNA quantification using RNase protection analysis¹⁶. IL-8 protein in amniotic fluid, BALF and homogenated lung tissue (right lower lobe) was measured by ELISA¹⁶. The minimum detection of the IL-8 assay was 12.5 pg/ml; inter- and intra-assay coefficients of variation were 8.96% and 8.84%, respectively.

For quantification of ureaplasma colonization, amniotic fluid samples were serially diluted (9 times; 10-fold dilutions) in 10B broth and incubated at 37°C. Ureaplasma growth (measured in color changing units; ccu) was detected by an alkaline shift and subsequent color change using phenol red indicator²⁰. For ureaplasma-negative amniotic fluid samples, DNA was extracted and PCR used to test for the presence of ureaplasmas, as described previously²⁶.

Data presentation and statistical analysis

Data are presented as mean $\pm$ SEM unless otherwise stated. Groups were compared using 3-way ANOVA with inoculate (ureaplasma or vehicle), maternal treatment (betamethasone or saline) and treatment-to-delivery interval (2 or 7 days) as factors (SPSS 13). Data were transformed if necessary to achieve normality.

Results

Of the 35 ewes randomized, one (vehicle at 86 days of pregnancy) aborted after betamethasone at 117 days. Another fetus (ureaplasmas at 86 days of pregnancy; betamethasone at 117 days)

had died *in utero* before Caesarean delivery. The remaining 33 animals were included in the analysis (Table 1).

Ureaplasmas were cultured from amniotic fluid samples, collected at delivery, from all but two of the animals (from the ureaplasma-and-7-day-betamethasone exposure group) that received an intra-amniotic ureaplasma injection; absence of ureaplasmas in these samples was confirmed by PCR. Ureaplasma color changing unit (ccu) counts were variable (from 10ccu/ml to 10^{10} ccu/ml); there was no effect of maternal betamethasone injection ($p=0.268$). Ureaplasmas were not cultured from any of the animals that received intra-amniotic vehicle injection.

Fetal body weight at delivery was not different between ureaplasma and vehicle groups ($P=0.673$). Maternal betamethasone injection (2 or 7 days before delivery) reduced fetal body weight by approximately 230g (10%) compared to control ($P=0.008$; Figure 1). Fetal lung weight was not affected by exposure to ureaplasmas ($P=0.179$) or betamethasone ($P=0.525$). Umbilical arterial blood gas measurements were variable, but within expected ranges: there was no effect of exposure to ureaplasmas or maternal betamethasone injection (data not shown; umbilical arterial pH values are shown in Table 1). The ratio of male-to-female fetuses was not different between groups (Table 1).

Fetal and maternal white blood cell counts (total and differential) were not affected by ureaplasma exposure or maternal betamethasone injection (Table 2). Inflammatory cells were not detected in BALF of any vehicle-exposed fetus but were present in BALF of 1/3-1/2 of the ureaplasma-exposed groups ($P=0.046$; Table 2). BALF inflammatory cell counts were not affected by betamethasone ($P=0.436$).

Interleukin-8 concentrations in amniotic fluid were higher in ureaplasma groups compared to vehicle ($p=0.049$; Table 3). In vehicle-exposed fetuses, maternal betamethasone injection increased IL-8 concentrations in lung tissue, BALF and amniotic fluid; but the effect was not statistically significant. Lung tissue levels of mRNA for IL-1 β , IL-6 or IL-8 were not altered by ureaplasma or betamethasone exposure (Table 3).

Lung compliance, as indicated by lung volume at a distending pressure of 40cmH₂O, was increased by both ureaplasma ($p=0.009$) and betamethasone ($p=0.042$) exposures (Figure 2). Lung volume at an airway pressure of zero, after deflation, was higher in ureaplasma-exposed fetuses than in vehicle groups ($p=0.007$) but was not affected by maternal betamethasone injection ($p=0.732$).

The concentration of Sat-PC in BALF was higher in ureaplasma-exposed fetuses than in vehicle groups ($p<0.001$; Figure 3). Maternal betamethasone injection 7 days before delivery, but not 2 days before, increased BALF Sat-PC concentrations, to a similar extent, in ureaplasma and vehicle groups ($p=0.001$). BALF Sat-PC concentrations were qualitatively lower in the ureaplasma-and-2-day-betamethasone group than in saline or 7-day-betamethasone groups but this effect was not statistically significant.

Comment

These studies represent the first experimental investigations, of which we are aware, of the efficacy of antenatal corticosteroids for improving preterm lung function in the presence of chronic amniotic fluid infection. Given the common use of antenatal corticosteroids in pregnancies in which intrauterine infection or inflammation is present⁹, the beneficial effects we have demonstrated on preterm lung function are reassuring. In this respect, our data are consistent with observations of human pregnancies¹¹. Our experiment was not designed to examine in detail the potential harmful effects of antenatal corticosteroids in the presence of

intrauterine infection, but the observations we have made are consistent with those of humans, that corticosteroid administration in the presence of intrauterine infection does not result in fulminating sepsis or any apparent deterioration in maternal or fetal wellbeing.

We have demonstrated previously an additive positive effect on preterm lung function of simultaneous maternal betamethasone and intra-amniotic LPS injection²²; our present results with ureaplasmas concur. In this previous study we noted also that intra-amniotic LPS injection nullified the fetal growth restricting effects of maternal betamethasone injection²². In our present study, inflammation induced by ureaplasma colonization did not have this growth preserving effect. This differential effect of LPS and ureaplasmas on glucocorticoid-induced fetal growth restriction likely relates to disparate effects of these 2 pro-inflammatory stimuli. Identification and investigation of these differences is beyond the scope of our studies thus far.

Unlike the consistent inflammatory and developmental effects on the fetal lungs of inflammation induced by intra-amniotic LPS injection¹⁵, inflammation induced by intra-amniotic ureaplasma injection, and effects on lung development, are variable: for example, some ureaplasma-exposed fetuses had an absence of inflammatory cells in BALF but high IL-8 levels and elevations in surfactant. We anticipate that at least some of the variability in effects of ureaplasma colonization that we observed is due to complicated interactions between the organisms themselves and the response of the host. Investigation of such interactions is necessary to understand ureaplasma pathogenicity. It is possible that the necessity of administration of MPA to prevent corticosteroid-induced preterm delivery affected ureaplasma-induced inflammation in our study because this form of progesterone has been shown to attenuate experimental intrauterine inflammation and associated preterm delivery²⁷. The design of our study, with MPA administered to all experimental groups, does not permit us to investigate this possibility formally. The variability in responsiveness to ureaplasmas observed by us is consistent with the disparate effects on human pregnancies of amniotic fluid ureaplasma colonisation⁶.

Augmentation of the effect on preterm lung function of inflammation by corticosteroids (or *vice versa*) is suggestive of independent mechanisms. Our pulmonary compliance (from pressure-volume curves) and surfactant lipid (Sat-PC) data together provide an indication of the underlying differential effects on preterm lung development of inflammation and corticosteroids. Ureaplasma exposure caused large increases in Sat-PC in BALF, whereas maternal betamethasone injection caused only modest increases, manifest only in the groups delivered 7 days after betamethasone injection. Despite these different effects on surfactant the functional consequences, in terms of lung compliance, were of similar magnitude (lung volume at 40cmH₂O was equivalent for vehicle-and-2-day-betamethasone, and ureaplasmas-and-saline groups). The functional benefit of betamethasone in the absence of appreciable surfactant increases can be attributed to changes in lung structure, such as thinning of the alveolar wall, which are induced by the corticosteroid within 2 days of maternal injection^{28–30}. Consistent with improvements in lung function being due to increased surfactant in the ureaplasma groups, lung volumes at low airway pressures were higher in these fetuses than in the vehicle groups.

We observed a (non-statistically significant) reduction in BALF Sat-PC in ureaplasma-exposed fetuses 2 days after maternal betamethasone treatment, similar to our previous observation of an attenuation of the surfactant response to LPS with simultaneous betamethasone administration²². Our previous experiments have established that inflammation induces profound increases in surfactant in the preterm lungs^{18, 29}. It is feasible that a short-term anti-inflammatory effect of corticosteroids may attenuate ongoing surfactant production by the inflamed fetal lungs; but this has not been formally investigated. This possibility has implications for clinical use of antenatal corticosteroids, since many women with intrauterine

infection/inflammation receive antenatal corticosteroids and deliver within 48 hours. In such a scenario, antenatal corticosteroids may induce lung structural change, but also abnormal surfactant production and/or secretion: these are the characteristics of bronchopulmonary dysplasia.

Similar to the pattern of effect of maternal betamethasone injection on BALF Sat-PC in ureaplasma-exposed animals, concentrations of IL-8 in BALF and amniotic fluid appeared to be initially decreased (2 days after injection), then increased (7 days after injection), by betamethasone. Further, in the absence of ureaplasmas, maternal betamethasone induced (non-statistically significant) increases in IL-8 in the amniotic fluid, BALF and fetal lung tissue. Ureaplasmas induce inconsistent patterns of inflammation, ranging from mild to severe, so our experiment was not ideally designed to investigate temporal effects of betamethasone on fetal inflammation; but our data support the likelihood that fetal inflammatory responses are modulated by corticosteroids in a time-dependent manner, and that this effect may contribute to both short and long term susceptibility to disease after birth²⁴.

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Figure 1.

Fetal body weights at 123 ± 2 (mean $\pm$ SD) days of gestation after exposure to ureaplasmas (black bars) or vehicle (white bars) from 86 ± 2 (mean $\pm$ SD) days, and either no maternal betamethasone exposure (none) or maternal betamethasone injection 2 days, or 7 days before delivery. Column height is group mean; error bars show SEM. Maternal betamethasone injection (either 2 or 7 days before delivery) reduced fetal body weight. * Indicates statistical difference ($p=0.008$; ANOVA) from corresponding control group (none).

Figure 2.

Lung pressure-volume relationship during deflation from an airway pressure of 40cmH₂O; for fetuses exposed to ureaplasmas (black symbols) or vehicle (white symbols) and either no maternal betamethasone exposure (circles) or maternal betamethasone injection 2 days (triangles), or 7 days (squares) before delivery. Lung volumes were elevated consistently by exposure to ureaplasmas. Maternal betamethasone injection increased lung volumes at high, but not low, airway pressures. * Indicates statistical difference ($p < 0.042$; ANOVA) from corresponding control group (none). # Indicates statistical difference (at 0cmH₂O, $p = 0.007$; at 40cmH₂O, $p < 0.001$; ANOVA) from vehicle groups.

Figure 3.

Bronchoalveolar lavage fluid saturated phosphatidylcholine (BALF Sat-PC) concentrations after exposure to ureaplasmas (black bars) or vehicle (white bars), and either no maternal betamethasone exposure (none) or maternal betamethasone injection 2 days, or 7 days before delivery. Column height is group mean; error bars show SEM. Exposure to ureaplasmas increased BALF Sat-PC concentrations. Maternal betamethasone injection 7, but not 2, days before delivery increased BALF Sat-PC concentrations in ureaplasma and vehicle groups. * Indicates statistical difference ($p=0.001$: AVNOA) from corresponding control group (none). # Indicates statistical difference ($p<0.001$: ANOVA) from vehicle groups.

Table 1
Experimental subjects

Experimental group		Number of singleton fetal lambs	Fetal sex ratio (male:female)	Umbilical arterial pH
Amniotic fluid inoculate	Betamethasone injection-to-delivery interval			
Vehicle	None	5	2:3	7.21 ± 0.05
	2 days	6	3:3	7.30 ± 0.03
	7 days	4	1:3	7.23 ± 0.04
<i>Ureaplasma parvum</i> (serovar 6)	None	6	4:2	7.23 ± 0.01
	2 days	6	3:3	7.28 ± 0.06
	7 days	6	4:2	7.23 ± 0.04

Table 2
Circulating maternal and fetal leukocytes and fetal pulmonary inflammatory cells

Experimental group		Maternal WBC count (10 ⁹ cells/ l)	Fetal WBC count (10 ⁹ cells/ l)	BALF inflammatory cell count(10 ⁶ cells/kg body weight)
Amniotic fluid inoculate	Betamethasone injection-to-delivery interval			
Vehicle	None	5.4 ± 0.2	3.9 ± 0.5	<0.01
	2 days	6.2 ± 1.0	3.4 ± 0.6	<0.01
	7 days	7.3 ± 0.8	7.4 ± 4.3	<0.01
<i>Ureaplasma parvum</i> (serovar 6)	None	4.9 ± 0.3	2.3 ± 0.4	30.1 ± 27.3 *
	2 days	5.4 ± 0.6	2.8 ± 0.6	18.8 ± 18.1 *
	7 days	5.7 ± 0.5	3.6 ± 0.5	57.8 ± 55.0 *

WBC, white blood cells; BALF, bronchoalveolar lavage fluid.

* Effect of ureaplasmas; p=0.046.

Table 3

Cytokines

Amniotic fluid inoculate	Experimental group		BALF (µg/ml)	IL-8 protein		Lung tissue (µg/kg body weight)	Messenger RNA in lung tissue ^J		
	Betamethasone injection-to-delivery interval			Amniotic fluid (ng/ml)			IL-1β	IL-6	IL-8
Vehicle	None		0	0		0.07 ± 0.01	1.00 ± 0.06	1.00 ± 0.07	1.00 ± 0.07
	2 days		0.24 ± 0.11	2.04 ± 1.20		0.70 ± 0.07	0.88 ± 0.06	1.00 ± 0.06	1.05 ± 0.07
	7 days		0.35 ± 0.13	1.84 ± 1.18		0.79 ± 0.06	-	-	-
<i>Ureaplasma parvum</i> (serovar 6)	None		0.64 ± 0.21	5.25 ± 0.66 [*]		0.20 ± 0.08	0.91 ± 0.09	0.96 ± 0.10	1.15 ± 0.10
	2 days		0.44 ± 0.18	4.66 ± 1.01 [*]		0.15 ± 0.03	1.14 ± 0.20	1.06 ± 0.15	1.34 ± 0.23
	7 days		0.99 ± 0.60	8.32 ± 0.56 [*]		0.33 ± 0.12	-	-	-

BALF, bronchoalveolar lavage fluid.

^J Relative to vehicle and saline controls.^{*} Effect of ureaplasmas; p=0.049.