Effects of prenatal ethanol exposure on the lungs of postnatal lambs

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Submitted 11 June 2010; accepted in final form 23 October 2010

Sozo F, Vela M, Stokes V, Kenna K, Meikle PJ, De Matteo R, Walker D, Brien J, Bocking A, Harding R. Effects of prenatal ethanol exposure on the lungs of postnatal lambs. Am J Physiol Lung Cell Mol Physiol 300: L139–L147, 2011. First published October 29, 2010; doi:10.1152/ajplung.00195.2010.—Prenatal ethanol exposure increases collagen deposition and alters surfactant protein (SP) expression and immune status in lungs of near-term fetal sheep. Our objectives were to determine (1) whether these prenatal effects of repeated gestational ethanol exposure persist after birth and (2) whether surfactant phospholipid composition is altered following prenatal ethanol exposure. Pregnant ewes were chronically catheterized at 90 days of gestational age (DGA) and given a 1-h daily infusion of ethanol (0.75 g/kg, n = 9) or saline (n = 7) from 95 to 135 DGA; ethanol administration ceased after 135 DGA. Lambs were born naturally at full term (146 ± 0.5 DGA). Lung tissue was examined at 9 wk postnatal age for alterations in structure, SP expression, and inflammation; bronchoalveolar lavage fluid was examined for alterations in surfactant phospholipid composition. At 134 DGA, surfactant phospholipid concentration in amniotic fluid was significantly reduced (P < 0.05) by ethanol exposure, and the composition was altered. In postnatal lambs, there were no significant differences between treatment groups in birth weight, postnatal growth, blood gas parameters, and lung weight, volume, tissue fraction, mean linear intercept, collagen content, proinflammatory cytokine gene expression, and bronchoalveolar lavage fluid surfactant phospholipid composition. Although SP-A, SP-B, and SP-C mRNA levels were not significantly different between treatment groups, SP-D mRNA levels were significantly greater (P < 0.05) in ethanol-treated animals; as SP-D has immunomodulatory roles, innate immunity may be altered. The adverse effects of daily ethanol exposure during late gestation on the fetal lung do not persist to 2 mo after birth, indicating that the developing lung is capable of repair.

WOMEN CONTINUE TO CONSUME alcohol during pregnancy, despite widespread educational information about its harmful effects on the fetus (8, 11). It is well established that chronic prenatal ethanol exposure can lead to a spectrum of growth and mental deficiencies, collectively termed fetal alcohol spectrum disorders (21). The effects of prenatal ethanol exposure on the developing brain are widely studied and well documented (34); however, less is known about the effects of prenatal ethanol exposure on the developing lungs.

Epidemiological studies have reported a significant increase in the number of congenital abnormalities of the respiratory tract in children born to women who had abused alcohol during pregnancy (4). Studies in rodents have also shown that exposure to high levels of ethanol in midgestation (41) or throughout pregnancy (23) decreases fetal lung growth. In contrast, exposure to a moderate level of ethanol during the later stages of gestation in sheep, when structural and functional maturation of the lungs occurs, does not alter fetal lung growth (37), although collagen deposition in the fetal lungs is increased and surfactant protein (SP) levels are reduced (28, 37).

It is well established that chronic ethanol exposure in adults increases the incidence and severity of acute respiratory distress syndrome (32). In addition to a reduction in surfactant levels, acute respiratory distress syndrome is characterized by inflammation and fibrosis of the lung parenchyma and an increased risk of pulmonary infection (2). Maternal alcohol consumption during pregnancy is also known to increase the risk of infection in neonates (12, 13). Furthermore, studies of children with fetal alcohol syndrome have reported an increased susceptibility to pneumonia and upper respiratory infections (25). Prenatal ethanol exposure has been found to decrease ciliary beat frequency, which could impair the clearance of pathogens from the respiratory tract (28), and also increase apoptosis of alveolar macrophages and decrease their function (15). Collectively, these studies suggest that prenatal ethanol exposure reduces the ability of the lungs to combat infection and clear pathogens.

Our laboratory recently reported the effects of repeated ethanol exposure during the third-trimester equivalent on fetal ovine lungs (37). A recent survey of drinking habits showed that ~50% of pregnant women consume alcohol in the third trimester of pregnancy (8). The ethanol dosage regimen used in our study was chosen to mimic human daily consumption of three to four standard drinks over 1 h. Using this regimen, we found that repeated ethanol exposure during late gestation results in increased collagen deposition and reduced SP and proinflammatory cytokine mRNA levels in the near-term fetal lung, with no alterations in lung growth or alveolarization (37). However, it is not known whether these prenatal effects of ethanol exposure persist after birth. If they do, functional development of the lungs could be impaired, contributing to respiratory illness after birth. Therefore, our principal aim was to determine whether the changes observed in the near-term fetal lung immediately following prenatal ethanol exposure persist after birth to 2 mo of postnatal age (PNA). We chose this time point, because at this age, sheep are prepubescent (i.e., similar to late childhood). A second aim was to determine the effects of prenatal ethanol exposure on the composition of pulmonary surfactant before and after birth.
METHODS

Experimental Procedure

All experimental procedures involving the use of animals were approved by the Monash University Animal Ethics Committee. Pregnant Border-Leicester × Merino ewes underwent aseptic surgery at 90 days of gestational age (DGA; full term ~147 days). Anesthesia was induced by an intravenous injection of thiopental sodium (1 g) and maintained by inhalation of 1–2% halothane in O₂-N₂O (70:30 vol/vol). Catheters were chronically inserted into a maternal carotid artery and jugular vein for blood sampling and ethanol or saline infusion, respectively. Antibiotics (ampicillin sodium) were administered to the amniotic sac (800 mg/4 ml) and fetus (200 mg/1 ml iv) for 3 days following surgery.

Pregnant ewes were given a daily 1-h intravenous infusion of ethanol (0.75 g/kg body wt, \( n = 9 \)) or an equivalent volume of saline \( (n = 7) \) from 95 to 135 DGA; fetuses were exposed to the same dose of ethanol via its transfer through the placental circulation. Ewes were allowed to deliver naturally at full term, and lambs were raised with their mothers until necropsy at 9 wk PNA. At 7 wk PNA, the lambs underwent brief aseptic surgery for the chronic insertion of vascular catheters for blood sampling. At 8 wk PNA, blood gases and blood chemistry were analyzed (model ABL700, Radiometer, Copenhagen, Denmark).

The lambs were euthanized at 9 wk PNA by an overdose of pentobarbital sodium (130 mg/kg iv). The lambs were weighed, and the lungs were removed and weighed. The left bronchus was ligated, and the left and right lungs were separated. Small portions of the left lung were snap-frozen in liquid nitrogen and stored at ~80°C for molecular analysis; care was taken to avoid major airways and blood vessels. The upper lobe of the right lung was isolated and cannulated for the collection of bronchoalveolar lavage fluid (BALF). Saline was infused via the cannula into the lobe until it was maximally expanded and then withdrawn; this was performed three times, and the final sample was collected as BALF. The BALF was centrifuged at 1,250 rpm for 7 min to separate the cellular component. The supernatant was collected and stored at ~80°C for surfactant phospholipid analysis. The right lung was then fixed via the trachea using 4% paraformaldehyde (in 0.1 M PBS, pH 7.4) at a distending pressure of 20 cmH₂O. Lung volume was estimated using the Cavalieri method (31). Randomly selected sections from the upper, middle, and lower right lung lobes were processed and embedded in paraffin for light microscopy analyses.

Morphometric Analysis

Paraffin-embedded sections of lung were stained with hematoxylin and eosin. For determination of tissue and air space fractions and the mean linear intercept, test grids were superimposed over projected images of the lung. Analyses were performed at a final magnification of ×400. Paraffin sections were also stained with the Gordon and Sweet reticular fiber stain, which stains type I and III collagen; the sections were counterstained with eosin. Analyses were performed at a final magnification of ×1,000.

Sections were viewed using a light microscope, and digital images were acquired using a Jenoptik camera attachment and ProRes CapturePro 2.6 software (Jenoptik). Images were analyzed using ImagePro Plus software (Media Cybernetics, Silver Spring, MD). Five nonoverlapping fields of view per section from three different regions of the lung (upper, middle, and lower lobes) per animal were analyzed for each analysis. The analysis was performed on the peripheral lung parenchyma, with care taken to avoid areas containing major airways or blood vessels. All measurements were made on coded slides by the same observer (MV), who was “blinded” to the experimental groups. For the collagen analysis, the area of tissue stained for collagen was expressed as a percentage of the total area of tissue for each field of view. This was then averaged for each animal and each experimental group.

Gene Expression Analysis

SP-A, SP-B, SP-C, SP-D, IL-1β, IL-6, IL-8, and TNF-α mRNA levels in lung tissue were measured using quantitative real-time PCR (qPCR), as previously described (37). Briefly, total RNA was extracted, treated with DNase (Qiagen), and then reverse-transcribed into cDNA (Maloney’s murine leukemia virus reverse transcriptase, RNase H Minus, Point Mutant Kit, Promoega, Madison, WI). qPCR was performed using a Real-Time Multiplexing System (Eppendorf) under previously described reaction conditions (37). Dissociation curves were performed for each qPCR experiment to ensure that a single PCR product had been amplified per primer set. Each sample was measured in triplicate, and a negative control sample that did not contain template cDNA was included in each run. The mRNA levels of each gene for each animal were normalized to the 18S rRNA values for that animal with use of the ΔΔCt method (where Ct is cycle threshold). Values are expressed relative to the mean gene mRNA levels in control lambs.

Surfactant Phospholipid Analysis

To determine the effects of ethanol exposure on fetal surfactant production and composition, the phospholipid content of amniotic fluid was assessed using samples obtained prior to necropsy (at 134 DGA) from our previous study (37). Amniotic fluid was collected via a catheter that was chronically implanted into the amniotic sac and exteriorized from the ewe; samples were stored at ~20°C until analyzed. We also assessed the phospholipid content of BALF obtained from 9-wk-old postnatal lambs at necropsy.

Phospholipids were extracted from the amniotic fluid samples and BALF supernatant (10 μl) with chloroform-methanol (2:1, 20 vol) following the addition of internal standards [100 pmol each of phosphatidylglycerol (PG) 17:0/17:0, phosphatidyserine (PS) 17:0/17:0, phosphatidycholine (PC) 13:0/13:0, and phosphatidylethanolamine (PE) 17:0/17:0 (Avanti Polar Lipids)]. Samples were vortexed, mixed for 10 min on a rotation mixer, sonicated for 30 min, and left to stand at room temperature for 20 min. Extracts were then centrifuged at 13,000 g for 10 min, and the supernatant was transferred to a 96-well plate and dried under nitrogen at 40°C. The samples were reconstituted in 50 μl of water-saturated n-butanol containing 10 mM NH₄COOH; then 50 μl of methanol containing 10 mM NH₄COOH was added. The plate was centrifuged at 3,000 g for 5 min, and the supernatant was transferred to glass sample vials for liquid chromatography-mass spectrometry.

Phospholipid analysis was performed by electrospray ionization-tandem mass spectrometry using a mass spectrometer (PE Sciex API 4000 Q/TRAP) with a turbo-ion spray source and Analyst 1.5 data system. Prior liquid chromatographic separation was performed on a Zorbax 2.1 mm C18 column (Zorbax) at 300 μl/min (50:20:30 and 75:20:25 tetrahydrofuran-methanol-water, respectively; both contained 10 mM NH₄COOH). Individual lipid species were quantified using scheduled multiple-reaction monitoring experiments were based on product ion of 184 mass-to-charge ratio \( (m/z) \) of [phosphocholine]⁻ for PC and neutral loss of 189 Da for PG, 141 Da for PE, 185 Da for PS, and 277 Da for PI. Each ion pair was monitored for 10–50 ms with a resolution of 0.7 atomic mass units at half-peak height and averaged from continuous scans over the elution period. Lipid concentrations were calculated by relation of the peak area of each species to the peak area of the corresponding internal standard (39). PI
species were related to the PE internal standard, and the higher response of the PE standard was corrected by a factor of 0.34. Total lipids of each class were calculated by summation of the individual lipid species.

The phospholipid class composition is expressed as a molar percentage of the total phospholipids measured. The molecular species composition within each phospholipid class is expressed as a molar percentage of its respective phospholipid class. Molecular species are denoted as follows: A\( + Bx + y\), where A and B are the number of carbon atoms in the fatty acid chains esterified at the sn-1 and sn-2 positions, respectively, and x and y are the number of double bonds in the fatty acid chains.

Statistical Analysis

Values are means ± SE. Student’s unpaired t-test was used to compare the mean values of data from the control and ethanol-exposed lambs. The Mann-Whitney rank sum test was used for analysis of data that were not normally distributed or had unequal variances. Two-way repeated-measures ANOVA was used for analysis of the postnatal growth of lambs. \( P < 0.05 \) was considered to be statistically significant.

RESULTS

Postnatal Body Growth and Morphometry

All lambs were born at full term (146 ± 1 and 145 ± 1 DGA for control and ethanol-exposed lambs, respectively). There was no difference in body weight at birth between the control and ethanol-exposed lambs (4.6 ± 0.2 and 4.3 ± 0.2 kg, respectively). There were no differences in postnatal growth between the control and ethanol-exposed lambs, as indicated by their percent mean weekly weight gain, crown-to-rump length, thoracic girth, forelimb length, and ponderal index (data not shown). At necropsy, there was no difference in body weight between the control and ethanol-exposed lambs (18.0 ± 2.0 and 18.6 ± 1.0 kg, respectively). Similarly, organ weights (absolute and adjusted for body weight) did not differ between treatment groups (see Supplemental Table S1 in Supplemental Material for this article, available online at the Journal website).

Arterial Blood Chemistry

At 8 wk PNA, there were no differences between the control and ethanol-exposed lambs in arterial pH, \( P_{CO_2} \), \( P_{O_2} \), and saturation of \( O_2 \), total hemoglobin, hematocrit, and \( HCO_3^- \), \( K^+ \), \( Na^+ \), \( Ca^{2+} \), \( Cl^- \), glucose, and lactate concentrations (data not presented).

Lung Weights

There was no significant difference in wet lung weight between the control and ethanol-exposed lambs (230.7 ± 22.1 and 252.5 ± 11.0 g, respectively) or wet lung weight relative to body weight (13.1 ± 0.6 and 13.7 ± 0.3 g/kg for control and ethanol-exposed lambs, respectively). Dry lung weight also did not differ between the control and ethanol-exposed lambs (40.4 ± 6.5 g (2.2 ± 0.2 g/kg) and 45.8 ± 4.0 g (2.5 ± 0.2 g/kg) for control and ethanol-exposed lambs, respectively).

Lung Parenchyma Morphometry

Lung volume at 20 cmH\(_2\)O was not significantly different between control and ethanol-exposed lambs (219.6 ± 14.6 and 222.7 ± 7.0 cm\(^3\), respectively), even when adjusted for body weight (12.8 ± 0.9 and 12.2 ± 0.5 cm\(^3\)/kg, respectively). There was no significant difference in the percentage of lung tissue between control and ethanol-exposed lambs (21.9 ± 0.9% and 24.5 ± 1.4%, respectively; Fig. 1). Correspondingly, the percentage of air space was not significantly different.
between the treatment groups (78.1 ± 0.9% and 75.5 ± 1.4% for control and ethanol-exposed lambs, respectively). The mean linear intercept was not significantly different between the control and ethanol-exposed lambs (130.0 ± 3.7 and 124.9 ± 3.9 μm, respectively; Fig. 1). There was also no significant difference in the areal percentage of collagen within the lung tissue of control and ethanol-exposed lambs (32.8 ± 1.0% and 35.3 ± 1.4%, respectively; Fig. 1).

**SP Gene Expression**

**Fetal sheep.** The concentration (P = 0.015) of total phospholipids measured was significantly lower in the amniotic fluid of 134 DGA ethanol-exposed than control fetuses (20,571 ± 3,118 vs. 31,847 ± 2,399 pmol/ml). The concentration of each phospholipid class measured was also significantly lower (P < 0.05) following ethanol exposure (Fig. 3). Prenatal ethanol exposure also significantly reduced (P < 0.05) the proportions of PS, PG, and PE relative to the total amount of phospholipids measured and tended to reduce the proportion of PI (P = 0.080; Fig. 3). Correspondingly, the proportion of PC was significantly greater (P = 0.011) in the amniotic fluid of ethanol-exposed fetuses. The concentration of the major molecular species of each phospholipid class was reduced, although their proportions were largely unaltered (see Supplemental Tables S2 and S3).

**Lung Proinflammatory Cytokine Gene Expression**

There were no significant differences in IL-1β (1.0 ± 0.2 and 1.0 ± 0.1 for control and ethanol-exposed lambs, respectively), IL-6 (1.0 ± 0.2 and 0.9 ± 0.3 for control and ethanol-exposed lambs, respectively), and IL-8 (1.0 ± 0.2 and 1.4 ± 0.3 for control and ethanol-exposed lambs, respectively) mRNA levels between the treatment groups (Fig. 5). TNF-α mRNA levels tended to be greater in ethanol-exposed than control lambs (1.4 ± 0.1 vs. 1.0 ± 0.2); however, this did not reach statistical significance (P = 0.093).

**Postnatal lambs.** In the 9-wk-old lambs, there were no significant differences between treatment groups in the proportions of the major classes of pulmonary surfactant phospholipids PS, PI, PC, PG, or PE relative to the total amount of phospholipids measured in BALF (Table 1). There were no significant differences in the proportions of the PC molecular species 30:0 +1, 32:0 +1, 32:1 +1, 34:1 +1, and 34:2 relative to the total PCs measured in the BALF of control and ethanol-exposed lambs (Fig. 4). There were no significant differences in the proportions of the PG molecular species 16:0/16:0, 16:0/16:1, 16:1/18:1, and 18:0/18:1 relative to the total PGs measured in the BALF of control and ethanol-exposed lambs (Fig. 4). The proportion of PG 16:0/18:1 tended to be lower in the BALF of the ethanol-exposed than control lambs (47.8 ± 1.9% vs. 52.5 ± 0.9%); however, this did not quite reach statistical significance (P = 0.067). The proportions of the PE molecular species 34:1, 34:2, 36:1, 36:2, or 36:3 relative to the total PEs measured in the BALF of control and ethanol-exposed groups were not significantly different (Fig. 4). The proportions of the PS and PI molecular species were not determined because of the low abundance of these classes within the total phospholipids detected in the BALF (<1%).

**Phospholipid Composition**

**Postnatal lambs.** In the 9-wk-old lambs, there were no significant differences between treatment groups in the proportions of the major classes of pulmonary surfactant phospholipids PS, PI, PC, PG, or PE relative to the total amount of phospholipids measured in BALF (Table 1). There were no significant differences in the proportions of the PC molecular species 30:0 +1, 32:0 +1, 32:1 +1, 34:1 +1, and 34:2 relative to the total PCs measured in the BALF of control and ethanol-exposed lambs (Fig. 4). There were no significant differences in the proportions of the PG molecular species 16:0/16:0, 16:0/16:1, 16:1/18:1, and 18:0/18:1 relative to the total PGs measured in the BALF of control and ethanol-exposed lambs (Fig. 4). The proportion of PG 16:0/18:1 tended to be lower in the BALF of the ethanol-exposed than control lambs (47.8 ± 1.9% vs. 52.5 ± 0.9%); however, this did not quite reach statistical significance (P = 0.067). The proportions of the PE molecular species 34:1, 34:2, 36:1, 36:2, or 36:3 relative to the total PEs measured in the BALF of control and ethanol-exposed groups were not significantly different (Fig. 4). The proportions of the PS and PI molecular species were not determined because of the low abundance of these classes within the total phospholipids detected in the BALF (<1%).

**Lung Proinflammatory Cytokine Gene Expression**

There were no significant differences in IL-1β (1.0 ± 0.2 and 1.0 ± 0.1 for control and ethanol-exposed lambs, respectively), IL-6 (1.0 ± 0.2 and 0.9 ± 0.3 for control and ethanol-exposed lambs, respectively), and IL-8 (1.0 ± 0.2 and 1.4 ± 0.3 for control and ethanol-exposed lambs, respectively) mRNA levels between the treatment groups (Fig. 5). TNF-α mRNA levels tended to be greater in ethanol-exposed than control lambs (1.4 ± 0.1 vs. 1.0 ± 0.2); however, this did not reach statistical significance (P = 0.093).
DISCUSSION

Previous studies have shown that prenatal ethanol exposure alters fetal lung development. In particular, it has been shown that prenatal ethanol exposure can alter fetal lung growth (23, 41), SP gene expression (28, 37), and immune status (12–17, 25, 37); our present study provides further evidence of reduced surfactant content and altered surfactant composition in ethanol-exposed fetuses. However, few studies have investigated whether these effects persist after birth. Our study of postnatal lambs shows that repeated ethanol exposure during the last

![Fig. 3. Surfactant phospholipid composition in amniotic fluid at 134 days of gestation. A and B: concentration and proportion of phospholipid classes phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC), phosphatidylglycerol (PG), and phosphatidylethanolamine (PE) in amniotic fluid from control fetuses (filled bars) and fetuses exposed to ethanol (open bars). Proportion of each phospholipid class is expressed as a percentage of the total phospholipid measured in amniotic fluid. *Significantly different (P < 0.05) from control.

Table 1. Proportions of the major classes of surfactant phospholipids in 9-wk-old lambs

<table>
<thead>
<tr>
<th>Phospholipid Class</th>
<th>Treatment Group</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>PC</td>
<td>85.1 ± 1.4</td>
</tr>
<tr>
<td>PG</td>
<td>10.9 ± 1.3</td>
</tr>
<tr>
<td>PE</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>PS</td>
<td>0.27 ± 0.06</td>
</tr>
<tr>
<td>PI</td>
<td>0.76 ± 0.08</td>
</tr>
</tbody>
</table>

Values are means ± SE, expressed as percentage of each phospholipid class relative to total amount of phospholipids measured in bronchoalveolar lavage fluid from control lambs and lambs exposed to ethanol prenatally. PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol.

![Fig. 4. Proportions of surfactant phospholipid molecular species in bronchoalveolar lavage fluid (BALK) from 9-wk-old lambs. A–C: proportions of the main phospholipid molecular species of PC, PG, and PE in BALF from control lambs (filled bars) and lambs prenatally exposed to ethanol (open bars). Proportion of each phospholipid species is expressed as a percentage of the total phospholipid class measured in BALF. No significant differences between treatment groups were observed.

41), SP gene expression (28, 37), and immune status (12–17, 25, 37); our present study provides further evidence of reduced surfactant content and altered surfactant composition in ethanol-exposed fetuses. However, few studies have investigated whether these effects persist after birth. Our study of postnatal lambs shows that repeated ethanol exposure during the last
third of gestation increases SP-D mRNA levels but does not alter lung growth, structure, surfactant phospholipid composition, or proinflammatory cytokine gene expression during early postnatal life.

Postnatal Growth and Physiological Status

Repeated exposure to moderate levels of ethanol during the last third of gestation did not affect prenatal or postnatal growth, as birth weights were unaffected and there were no significant differences between groups in weekly weight gain or body proportions. These findings are consistent with the lack of fetal growth restriction previously reported using our model of daily prenatal ethanol exposure (37). In contrast, studies in rodents have found that prenatal ethanol exposure reduces fetal body weight and lung growth (23, 41). Differences between these studies may be due to differences between species or, more likely, differences in the timing and dose of ethanol exposure. It is known that the degree of growth restriction in humans prenatally exposed to ethanol is related to the degree of ethanol exposure (1, 35). For example, individuals diagnosed with fetal alcohol syndrome and, therefore, exposed to high levels of alcohol have a low birth weight (1), and this growth restriction persists into childhood (26) and adulthood (38). Furthermore, fetal sheep that were exposed to high levels of ethanol throughout gestation also have significant growth restriction compared with controls (33). Conversely, growth was less restricted in offspring of women who consumed large amounts of alcohol during the early stages of their pregnancy but then abstained or markedly reduced their alcohol consumption prior to the third trimester than in offspring of women who continued to consume large amounts of alcohol throughout pregnancy (35).

Lung Morphometry

Repeated ethanol exposure during late gestation did not affect lung growth; nor did it alter tissue and air space fraction or mean linear intercept after birth. Although these morphometric measures are not a direct indication of the number of alveoli, they would be expected to be altered if alveolarization was impaired. Therefore, our study suggests that prenatal ethanol exposure does not affect alveolarization after birth. Arterial blood gas parameters were also not different in postnatal lambs exposed prenatally to ethanol, indicating that pulmonary gas exchange had not been impaired by prenatal ethanol exposure. These findings are in agreement with the lack of effects of prenatal ethanol exposure on alveolarization in the near-term fetal sheep lung (37). In contrast to the increase in the deposition of collagen within the fetal lung following prenatal ethanol exposure (37), there was no effect on lung collagen deposition 2 mo after birth. In our present and previous (37) studies, ethanol was administered to the pregnant ewes in the ethanol-exposed group daily between DGA 95 and 135. However, in the present study, there was a period before birth (from 136 to 146 ± 0.5 DGA) when fetuses were not exposed to ethanol. Furthermore, the alveolar stage of lung development in sheep continues until ∼6 mo PNA (3). Therefore, alveolarization was ongoing during this ethanol-free period in utero and after birth. This continuing development and remodeling of the lung parenchyma without the influence of ethanol may have resulted in the degradation of the excess collagen, although the mechanisms involved are unknown.

Pulmonary Surfactant Composition

Surfactant consists of ∼90% lipids, ∼90–95% of which are phospholipids and ∼5–10% are neutral lipids (e.g., choles-
terol). Phospholipids within surfactant act to reduce the surface tension at the air-liquid interface within the lung and, thus, help prevent alveolar collapse at end expiration. Alterations in the composition of the phospholipid component of surfactant can alter surfactant properties; for example, the acidic phospholipids PG and PI specifically contribute to adsorption (30), PCs have a major role in lowering surface tension during compression as well as in surfactant film respreading (40), and saturation of the fatty acid chains affects the fluidity of surfactant. The concentration of total phospholipids and each phospholipid class measured in amniotic fluid was significantly lower following ethanol exposure; the phospholipid content of amniotic fluid is representative of the phospholipid composition of fetal pulmonary surfactant that has been secreted in lung liquid. The absolute concentration of phospholipids in the amniotic fluid, however, will be influenced by many factors, including lung liquid secretion rates, fetal swallowing, and fetal urine production. It is therefore possible that the phospholipid concentration could be altered by fetal alcohol exposure by changes in any of these functions. Our findings are in agreement with previous studies that have shown a reduction in SP mRNA levels following fetal ethanol exposure (28, 37). Furthermore, it has been shown that women who continued to consume large amounts of alcohol throughout pregnancy did not show the expected increases in PC and PG in amniotic fluid that were observed in those who abstained from alcohol consumption (19). In the present study, the phospholipid composition of amniotic fluid was also significantly altered in fetuses exposed to ethanol. Specifically, the proportion of PC was greater in the amniotic fluid of ethanol-exposed fetuses, while the proportions of PS, PG, and PE were lower. As PCs have the greatest ability to reduce surface tension at the air-liquid interface, an increase in the proportion of PCs could be a compensatory response to the reduction in total phospholipid concentration. Overall, these studies suggest that the amount and composition of surfactant are altered following prenatal ethanol exposure, and this could adversely affect respiratory function at birth. In the present study, however, there were no significant differences in surfactant phospholipid composition in postnatal lambs, suggesting that the functional properties of surfactant normalized between the last ethanol exposure and 9 wk PNA.

Pulmonary surfactant contains ~10% protein, made up of SP-A, SP-B, SP-C, and SP-D. SP-B and SP-C are hydrophobic and help reduce the surface tension at the air-liquid interface. Conversely, SP-A and SP-D are hydrophilic and primarily have immunoregulatory roles, including binding and opsonizing microorganisms to make them more susceptible to phagocytosis. Previous studies have found that ethanol exposure decreases pulmonary surfactant production and increases apoptosis of type II alveolar epithelial cells, which produce and secrete surfactant (5, 6, 18, 20). Furthermore, studies in fetal sheep have found that ethanol exposure decreases SP mRNA levels (28, 37). In the present study, however, there were no significant differences in SP-A, SP-B, or SP-C gene expression, suggesting that the effects of prenatal ethanol exposure do not persist after birth. These findings are similar to those of a study in fetal sheep that showed a reduction in SP-A mRNA levels immediately following ethanol exposure late in gestation (at 138 DGA) but no change at full term (at 147 DGA) (28). Therefore, it appears that further maturation of the lung or removal of the influence of ethanol allows the lung to repair the alterations in surfactant composition caused by ethanol exposure.

Despite the lack of effect of prenatal ethanol exposure on SP-A, SP-B, and SP-C mRNA levels after birth, SP-D mRNA levels were significantly increased. This increase could be a compensatory response to the decreased SP expression in the fetal lungs or may be associated with an increased proinflammatory state. For example, SP-D expression is stimulated by an upregulation of proinflammatory cytokines (22). Alternatively, an increase in SP-D expression could indicate an enhanced ability to protect the lung from microorganisms and, therefore, could be beneficial. Further studies are required to determine the functional consequences of an increase in SP-D expression; however, postnatal pulmonary immunity (9) and surfactant phospholipid homeostasis (27) may be adversely affected.

**Immune Status**

Previous studies have shown that maternal alcohol consumption increases the susceptibility of preterm and full-term neonates to infection (12, 13). In particular, there is evidence that maternal alcohol consumption increases the incidence of respiratory infections, such as pneumonia, in offspring, suggesting that the developing lung is specifically susceptible to the effects of ethanol (25). Furthermore, the altered immune function attributable to in utero alcohol exposure persists into childhood (25) and adulthood (42). Animal models of fetal ethanol exposure also suggest that immune system development is compromised, and this can persist into childhood and adulthood (7, 17, 24, 36). A recent study found that mice prenatally exposed to ethanol exhibit enhanced disease severity when infected with influenza virus in adulthood, and more time was taken to clear the virus from lungs of ethanol-exposed than control mice (29). This increased risk of infection could be partly due to a reduction in proinflammatory cytokine levels or a reduction in inflammatory cell number or function. Indeed, a reduction in proinflammatory cytokines in response to alcohol consumption has been demonstrated in adults (10) and fetuses (37). Fetuses and newborns exposed to ethanol during pregnancy have been shown to have impaired alveolar macrophage function, both basally and in response to infection (14–16). In the present study, however, the suppressive effect of prenatal ethanol exposure on proinflammatory cytokine expression did not persist after birth. Although this could indicate a restoration of the innate immune system to normal, the pulmonary response to infection may still be altered and requires further study. Furthermore, proinflammatory cytokine protein levels may be altered by a posttranscriptional mechanism; in addition, the number or function of the inflammatory cells on which these cytokines act may also be altered.

**Study Limitations**

In our study, the fetuses were not exposed to ethanol for the last 10–12 days of gestation. Therefore, although our study indicates that the immediate effects of prenatal ethanol exposure on the developing lung do not persist after birth, this ethanol-free period in utero could have contributed to the lung repair observed. It is not known whether the effects of ethanol exposure on the lung would differ if the exposure had continued until full term. Since the lung continues to develop after

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birth, however, it is possible that this period of remodeling could also have contributed to lung repair, although the exact mechanisms are unknown. Furthermore, it is likely that a higher level of ethanol exposure during pregnancy could have persistent adverse effects on the lung.

Conclusions

Repeated ethanol exposure during late gestation alters collagen deposition, surfactant composition, and proinflammatory cytokine gene expression in the near-term fetal lung. These effects, however, do not persist to 2 mo PNA, suggesting that the developing lung has the capacity to repair alterations in development and immune status in the absence of further ethanol exposure.

ACKNOWLEDGMENTS

We acknowledge the expert technical assistance of Natasha Blash, Alex Satragno, and Jonathan Bensley. We also acknowledge the expert technical assistance of Jacqui Weir (Blood and DNA Profiling Facility, Baker IDI) and the advice of Sandra Orgeig in the analysis of the surfactant phospholipid data.

GRANTS

This work was supported by the National Health and Medical Research Council of Australia and the Canadian Institutes of Health Research.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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