Repeated ethanol exposure during late gestation alters the maturation and innate immune status of the ovine fetal lung


1Department of Anatomy and Developmental Biology, Monash University, Melbourne, Victoria, Australia; 2Department of Medical Biosciences, University of the Western Cape, Bellville, South Africa; 3Department of Physiology, Monash University, Melbourne, Victoria, Australia; 4Department of Obstetrics and Gynaecology, University of Toronto, Toronto, Ontario, Canada; and 5Department of Pharmacology and Toxicology, Queen’s University, Kingston, Ontario, Canada

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Sozo F, O’Day L, Maritz G, Kenna K, Stacy V, Brew N, Walker D, Bocking A, Brien J, Harding R. Repeated ethanol exposure during late gestation alters the maturation and innate immune status of the ovine fetal lung. Am J Physiol Lung Cell Mol Physiol 296: L510–L518, 2009. First published December 26, 2008; doi:10.1152/ajplung.90532.2008.—Little is known about the effects of fetal ethanol exposure on lung development. Our aim was to determine the effects of repeated ethanol exposure during late gestation on fetal lung growth, maturation, and inflammatory status. Pregnant ewes were chronically catheterized at 91 days of gestational age (DGA; term ∼147 days). From 95-133 DGA, ewes were given a 1-h daily infusion of either 0.75 g ethanol/kg (n = 9) or saline (n = 8), with tissue collection at 134 DGA. Fetal lungs were examined for changes in tissue growth, structure, maturation, inflammation, and oxidative stress. Between treatment groups, there were no differences in lung weight, DNA and protein contents, percent proliferating and apoptotic cells, tissue and air-space fractions, alveolar number and mean linear intercept, septal thickness, type-II cell number and elastin content. Ethanol exposure caused a 75% increase in pulmonary collagen I α1 mRNA levels (P < 0.05) and a significant increase in collagen deposition. Surfactant protein (SP)-A and SP-B mRNA levels were approximately one third of control levels following ethanol exposure (P < 0.05). The mRNA levels of the proinflammatory cytokines interleukin (IL)-1β and IL-8 were also lower (P < 0.05) in ethanol-exposed fetuses compared with controls. Pulmonary malondialdehyde levels tended to be increased (P = 0.07) in ethanol-exposed fetuses. Daily exposure of the fetus to ethanol during the last third of gestation alters extracellular matrix deposition and surfactant protein gene expression, which could increase the risk of respiratory distress syndrome after birth. Changes to the innate immune status of the fetus could increase the susceptibility of the neonatal lungs to infection.

lung growth; lung morphometry; surfactant proteins; proinflammatory cytokines

ALCOHOL (ETHANOL) CONSUMPTION during pregnancy remains a significant health problem in many countries, especially among some indigenous communities. Recent figures show that many women consume alcohol during pregnancy in developed countries; for example, it has been reported that 30% of American women (19), 59% of Australian women (15), and 30–60% of European women (13, 16, 21, 26, 35, 38) drink alcohol at some stage during their pregnancy. Prenatal exposure to ethanol can result in the fetal alcohol syndrome (FAS) or fetal alcohol spectrum disorders (FASD), which are characterized by growth restriction, brain dysmorphology and dysfunction, in addition to craniofacial anomalies in the case of FAS (31, 34). The effects of fetal ethanol exposure on brain development are well documented (48); however, less is known about the effects of prenatal ethanol exposure on other organs, in particular, the developing lungs. To the best of our knowledge, there are no data on the effects of maternal alcohol consumption on fetal lung development in humans. However, alcohol consumption during pregnancy is known to increase the risk of low birth weight due to both preterm birth and intrauterine growth restriction (18). Because low birth weight is associated with an increased incidence of respiratory distress in the neonate and maternal alcohol consumption increases the risk of neonatal infection (4, 22), it is possible that prenatal ethanol exposure contributes to respiratory difficulties after birth.

It is well established that the lungs of adults can be affected by prolonged ethanol exposure. Alcoholic adults are known to have an increased risk of acute respiratory distress syndrome (ARDS) and pulmonary infections (1, 41). Furthermore, adult lungs exposed to ethanol have been shown to have increased type-II alveolar epithelial cell apoptosis, as well as decreased surfactant synthesis and expression of proinflammatory cytokines (7, 17, 27–29). If similar effects occurred in the fetus, functional development of the lungs could be impaired, contributing to respiratory distress after birth. Studies in rodents suggest inhibitory effects of prenatal ethanol exposure on lung growth (33, 50), but there is little information on fetal lung maturation in a species in which lung development during pregnancy more closely resembles that in humans. In particular, there is little information on the effects of prenatal ethanol exposure on lung structure, pulmonary surfactant, and the innate immune status of the lung before birth.

Our objective was to determine the effects of repeated ethanol exposure on the fetal lungs during the third-trimester equivalent, when structural and functional maturation of the lungs occurs. We have used sheep because the stages of lung development in the fetus are similar to those of humans, in which a large proportion of lung development, including the onset of alveolarization, occurs before birth (2, 9). The repeated maternal alcohol dosage regimen, involving daily 1-h ethanol infusions, was used to mimic human daily drinking of 3–4 standard drinks over 1 h (40). Alcohol readily passes in...
both directions across the sheep placenta, and its clearance from the maternal-fetal unit is predominantly regulated by its metabolism to acetaldehyde in the maternal liver; this is comparable to the metabolism of ethanol in humans (6, 14). In the present study, we examined the effects of repeated ethanol exposure during the third-trimester equivalent on fetal lung growth, parenchymal architecture, and the surfactant system. Because there is evidence that ethanol can affect proinflammatory cytokines in the lung (17, 27), we also examined the expression of major proinflammatory cytokines.

**METHODS**

**Experimental procedure.** Animal procedures were approved by the Monash University Animal Ethics Committee. Pregnant Border-Leicester × Merino ewes underwent aseptic surgery at 91 days of gestational age (DGA; term ~147 days). Anesthesia was induced by an intravenous injection of thiopental sodium (1 g) and was maintained by inhalation of 1–2% halothane in O2-N2O (50:50 vol/vol). Catheters were inserted chronically into a maternal carotid artery and jugular vein for blood sampling and ethanol or saline infusion, respectively. Pregnant ewes received daily 1-h intravenous infusions of either 0.75 g ethanol/kg body wt (n = 9) or saline (n = 8) from 95-124 DGA; fetuses received ethanol via the placenta. At 126 DGA, the ewes again underwent surgery for the implantation of catheters into a fetal brachial artery and the amniotic sac. The daily maternal infusion of ethanol or saline resumed on 127 DGA and continued until 133 DGA. Maternal and fetal arterial blood samples were taken before each infusion and at 1, 2, 4, 6, 8, 10, and 24 h postinfusion on gestational days 131–133. Blood samples were analyzed for plasma ethanol concentration (PEC) using the Dade Behring Dimension Rxl Clinical Chemistry System. Fetal blood samples were also analyzed for arterial blood gases and glucose and lactate concentrations to determine the physiological state of the fetus.

The pregnant ewes and fetuses were humanely killed at 134 DGA by an intravenous overdose of pentobarbital sodium (130 mg/kg), which was administered to the ewe. The fetuses were weighed and the lungs removed and weighed. The left bronchus was then ligated, and the left and right lungs were separated. Small portions (avoiding major airways and blood vessels) of the left lung were snap frozen in liquid nitrogen and stored at −70°C for molecular analysis. The right lung was fixed via the trachea using 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4) and at a distending pressure of 20 cmH2O. Sections were then processed and paraffin embedded for light microscopy analyses.

**DNA and protein assays.** Pulmonary DNA and soluble protein concentrations were determined using an established fluorometric DNA assay and a standard colorimetric protein assay (Bio-Rad), respectively (30, 44). DNA standards were prepared using salmon testes DNA (Sigma) diluted in sodium phosphate buffer (3 M NaCl, 0.05 M Na2HPO4, 0.05 M NaH2PO4·2H2O, 0.002 M EDTA, pH 7.4) to achieve concentrations of 100, 50, 25, 12.5, 6.25, and 0 μg/ml. Protein standards were prepared using bovine serum albumin (A-7888, Sigma) diluted in distilled water to achieve concentrations of 100, 50, 25, 12.5, 6.25, and 0 μg/ml.

**Immunohistochemical analysis.** Proliferating cells were identified by performing immunohistochemistry using an antibody against the Ki67 protein (1:100, 90-min incubation, mouse anti-human monoclonal antibody; DakoCytomation). The Ki67 protein is expressed during all active phases of the cell cycle but is absent in resting cells and is therefore a suitable marker of cell proliferation (25). Apoptotic cells were identified by performing immunohistochemistry using an antibody against the caspase-3 protein (1:1,000, 2-h incubation at 4°C, rabbit anti-mouse/human antibody; R&D Systems).

Randomly selected paraffin-embedded lung sections (5 μm) were incubated at 60°C for ~2 h, deparaffinized in xylene, rehydrated in graded ethanol washes, and washed in phosphate-buffered saline. For Ki67 immunohistochemistry, tissue sections were boiled for 20 min in a microwave oven (on high power) in an antigen retrieval solution (0.01 M sodium citrate, pH 6.0). For caspase-3 immunohistochemistry, tissue sections were boiled three times for 5 min each in 0.01 M citric acid (pH 6.0). An EnVision + Dual Link System-HRP (DAB + ) immunohistochemistry kit (DakoCytomation) was used to visualize positively stained nuclei. Sections were counterstained with hematoxylin. Specificity of immunostaining was confirmed by omission of the primary antibody.

Sections were examined using light microscopy, and digital images were acquired and analyzed using ImagePro Plus (Media Cybernetics, Silver Spring, MD). Analyses were performed at a final magnification of ×1,000. Five nonoverlapping fields of view per section from two to three sections (from different regions of the lung) per animal were analyzed. For each field of view, the number of positively stained cells and the total number of cells were counted. For each animal, the number of positively stained cells was then expressed as a percentage of the total number of cells counted. This was then averaged for each experimental group. The analysis was performed on the peripheral lung parenchyma, avoiding areas containing major airways or blood vessels.

**Morphometric analysis.** Paraffin-embedded sections of lung tissue were stained with hematoxylin and eosin. Measurements were made by superimposing test grids over projected images of the lung. Measurements were made on coded slides by the same observer (G. Maritz), who was blinded to the experimental groups. Tissue and air-space fractions, alveolar number, the mean linear intercept, or average distance between alveolar walls (Lm), and septal thickness were determined. The total number of alveoli (Nₐ) was determined using the following equation: Nₐ = Vₐ × Vₐ/Vₐ, where Vₐ is the percentage air-space of the lung and Vₐ is the mean alveolar volume, computed as Lm × π/3. Lung volume (Vₐ) was estimated using the Cavalieri method (39). Analyses were performed at a final magnification of ×400. At least 15 nonoverlapping fields of view per section from two to three sections (from different regions of the lung) per animal were analyzed.

The number of type-II cells was counted using a Zeiss photo reticle MC 2.5/d = 26 mm eyepiece. Type-II cells were identified as rounded or cuboidal cells, and only cells that had a visible nucleus and formed part of an alveolar wall were counted. Type-II cells that were inside the boundaries of the reticule and those that were on the lower and left-hand boundaries were counted, whereas type-II cells that were on the upper and right-hand boundaries were excluded. At least five nonoverlapping fields of view per section from two to three sections (from different regions of the lung) per animal were analyzed.

Type-II cell numbers were expressed per 10 mm² of lung tissue. **Elastin and collagen analysis.** Paraffin-embedded sections were stained for elastin using the Hart’s resorcin-fuchsin stain and counterstained with tartrazine (0.25%) in saturated picric acid. For the collagen analysis, paraffin-embedded sections were stained using the Gordon and Sweet reticular fiber stain, which stains collagen types I and III; the sections were counterstained with eosin. These sections were stained for elastin using the Hart’s resorcin-fuchsin stain and counterstained with tartrazine (0.25%) in saturated picric acid. The Ki67 protein (1:100, 90-min incubation, mouse anti-human monoclonal antibody; DakoCytomation). The Ki67 protein is expressed during all active phases of the cell cycle but is absent in resting cells and is therefore a suitable marker of cell proliferation (25). Apoptotic cells were identified by performing immunohistochemistry using an antibody against the caspase-3 protein (1:1,000, 2-h incubation at 4°C, rabbit anti-mouse/human antibody; R&D Systems).

**Quantitative histomorphometry.** The total number of alveoli in each field of view was counted. This was then averaged for each animal, the number of positively stained cells was then expressed as a percentage of the total number of cells counted. This was then averaged for each experimental group. The analysis was performed on the peripheral lung parenchyma, avoiding areas containing major airways or blood vessels.

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quantitative real-time polymerase chain reaction (qRT-PCR). Primers to amplify each gene were designed on the basis of ovine sequences (Table 1). Primers for the amplification of the housekeeping gene 18S (Table 1) were used to account for minor differences in sample preparation between animals.

Total RNA was extracted, DNase treated (Qiagen), and reverse transcribed into cDNA (M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant Kit; Promega). qRT-PCR was performed using a Realplex Real-Time Multiplexing System (Eppendorf) using reactions that contained cDNA template, forward and reverse primer (Table 1), SYBR green (Platinum SYBR Green qPCR SuperMix-UDG; Invitrogen Life Technologies), and nuclease-free water. The thermal profile used to amplify the PCR products included an initial 2-min incubation at 95°C, followed by 50 cycles of denaturation at 95°C for 3 s, annealing at 58/59/60°C (depending on the primers for each gene; see Table 1) for 20 s, and elongation at 72°C for 20 s. The fluorescence readings were recorded after each 72°C step. Dissociation curves were performed after each PCR run to ensure that a single PCR product had been amplified per primer set. Each sample was measured in triplicate, and a control sample, containing no template, was included in each run. The mRNA levels of each gene for each animal were normalized to the 18S rRNA values for that animal, was included to the mean gene mRNA levels in control fetuses.

Malondialdehyde assay. Malondialdehyde (MDA) levels were used as a measure of lipid peroxidation and, therefore, oxidative stress. The MDA method was based on measuring the absorbance of thiobarbituric acid (TBA)-MDA, as previously described (45). MDA standards were prepared using 1,3,3,3,6,6,7,7,8,8,8-decane-dioic acid (pH 7.4). The homogenate (1:10) were mixed with sodium dodecyl sulfate and acetic acid (pH 3.5) before centrifugation at 10,000 g for 15 min. The supernatant was collected, mixed with an aqueous solution of TBA, and heated at 95°C for 60 min. The absorbance of each sample was then measured at a wavelength of 532 nm. Samples in which the addition of TBA was omitted acted as blanks. A standard Lowry protein assay was conducted on the same lung homogenates, and MDA levels are expressed in nmol/mg protein.

Statistical analysis. Results are presented as means (SD). Student’s unpaired t-test was used to compare mean values of data from the control and ethanol-exposed fetuses. The COLIA1, COLIA2, SP-A, IL-1β, IL-6, and IL-8 gene expression data were normalized by log10 transformation. The lung volume, collagen content, and SP-D gene expression data were analyzed using the Mann-Whitney rank sum test. For the elastin analysis, a nested ANOVA design, which included treatment, lobe, and field of view as factors, was used to detect differences between groups. P < 0.05 was considered to be statistically significant.

Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Genbank Accession No.</th>
<th>Tm, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>F: GTCTGATGGCTGCCAGCATGATTC&lt;br&gt;R: AAACATGCTGACCCGACACTAGCC</td>
<td>X01117</td>
<td>60</td>
</tr>
<tr>
<td>COLIA1</td>
<td>F: AGACATCCACAGCACCAGC&lt;br&gt;R: AAGATCAGCTGTCACGGACA</td>
<td>AF129287</td>
<td>60</td>
</tr>
<tr>
<td>COLIA2</td>
<td>F: GGGTCAGCTGGAGACATTCC&lt;br&gt;R: TCTCCTACACGACATGCTTC</td>
<td>EF114225</td>
<td>59</td>
</tr>
<tr>
<td>COLIII</td>
<td>F: GTCTGCGAGAAGATGTTTGA&lt;br&gt;R: CAGACGAGCGGAGGAGAGAG</td>
<td>DQ239680</td>
<td>58</td>
</tr>
<tr>
<td>IL-1β</td>
<td>F: GGAGAGGTCCTGTGTACTG&lt;br&gt;R: CTAGGAGAGGAGGAGGAGAG</td>
<td>NM_001009465</td>
<td>60</td>
</tr>
<tr>
<td>IL-6</td>
<td>F: GCAGGAGTTATCATCATTG&lt;br&gt;R: CCCAGGAACTCAGCAATTGA</td>
<td>NM_001009392</td>
<td>59</td>
</tr>
<tr>
<td>IL-8</td>
<td>F: GCCAGAAGTAGGCGCAATGA&lt;br&gt;R: TGACACATTCCACACCAAGCA</td>
<td>NM_001009401</td>
<td>59</td>
</tr>
<tr>
<td>TNF-α</td>
<td>F: GGATTCTGCGACCTGCTCTCTTTG&lt;br&gt;R: GTACCTGAAGTGTGGGCAAG</td>
<td>NM_001024860</td>
<td>60</td>
</tr>
<tr>
<td>SP-A</td>
<td>F: CATGAAAACTCTCGAGTGCA&lt;br&gt;R: GCCGATTGAGGAGGAGGACG</td>
<td>NM_001009728</td>
<td>60</td>
</tr>
<tr>
<td>SP-B</td>
<td>F: GTCTCCTGCTGGAGACATG&lt;br&gt;R: GGAGAGTTCTGCTGTGCTAG</td>
<td>AF107544</td>
<td>59</td>
</tr>
<tr>
<td>SP-C</td>
<td>F: GTGAGAGACTCTCTGAGA&lt;br&gt;R: TCTGAGGAGACGTGACACTGG</td>
<td>NM_001009729</td>
<td>58</td>
</tr>
<tr>
<td>SP-D</td>
<td>F: ATGAGAGAGAGCAGAGGGA&lt;br&gt;R: GCCGATTGAGGAGGAGGACG</td>
<td>AJ133002</td>
<td>59</td>
</tr>
</tbody>
</table>

Sequences for each forward (F) and reverse (R) primer (5'-3') used to amplify each gene of interest. Primer sequences were designed on the basis of the nucleotide sequence that corresponds to the listed Genbank accession number. Annealing temperature (Tm) for each gene amplified during real-time PCR is also shown. COLIA1, collagen I α1; COLIII, collagen III; SP, surfactant protein.
but this was not significantly different (P = 0.083).

**Lung proinflammatory cytokine gene expression.** Fetal IL-1β mRNA levels were significantly lower (P = 0.031) in ethanol-exposed fetuses [0.33 (SD 0.20)] than in controls [1.00 (SD 0.74)] (Fig. 3). IL-6 mRNA levels in ethanol-exposed fetuses were 0.72 (SD 1.00) of control levels [1.00 (SD 0.91)], this was not statistically significant. The gene expression of IL-8 was significantly reduced (P = 0.042) by ethanol exposure [control: 1.00 (SD 1.33); ethanol: 0.14 (SD 0.28)]. TNF-α mRNA levels in ethanol-exposed fetuses were 0.41 (SD 0.28) of control levels [1.00 (SD 0.91)], however, this difference was not significant (P = 0.103).

**Lung oxidative stress.** MDA levels in the lungs of ethanol-exposed fetuses [0.58 nmol/mg protein (SD 0.20)] tended to be greater (P = 0.070) than in control fetuses [0.40 nmol/mg protein (SD 0.17)]; however, this did not reach statistical significance.

**DISCUSSION**

Few studies have investigated the effects of maternal alcohol consumption on the developing lung. In particular, there is very little information on the effects of repeated ethanol exposure on fetal lung growth, maturation, and inflammation in a species in which the prenatal development of the lung resembles that of humans. To the best of our knowledge, this is the first study to show that repeated ethanol exposure during the last third of gestation increases collagen expression and deposition and decreases surfactant protein and proinflammatory cytokine expression in the fetal lung, with no alterations in fetal body weight, lung growth, or lung structure.

**Lung growth.** Previous studies in rodents have shown that ethanol exposure during gestation causes a marked reduction in lung weight and RNA, DNA, and protein contents in the fetal lung (33, 50). In contrast, repeated ethanol exposure during late gestation in the present study did not alter fetal body weight or lung weight, lung DNA or protein contents or lung cell proliferation or apoptosis. These differences may be attributed to the timing and dose of ethanol exposure or differences between species. For example, in the present study, a daily dose of ethanol was infused during a 1-h period into the maternal circulation during the last third of gestation, simulating a “binge” pattern of drinking. Each daily infusion produced a rise in PEC to a maximum of 0.12 g/dl and 0.11 g/dl in the ewe and fetus, respectively, which returned to control baseline over an 8-h period. In rodent studies, pregnant dams were either continuously exposed to high levels of ethanol via their drinking water throughout pregnancy (33) or to high levels of ethanol over a 3-day period during midgestation (50). It is likely that exposure to high ethanol concentrations in early pregnancy, as in many rodent studies, reduces body and lung growth, but repeated ethanol exposure late in gestation, as in our study, does not.

**Lung parenchyma morphometry.** It has been suggested that prenatal ethanol exposure causes a delay in lung development (50). However, no previous studies have investigated the effects of prenatal ethanol exposure on alveolar structure using detailed morphometric techniques. Ours is the first study to show that repeated ethanol exposure during the period of alveolarization does not significantly alter the percentage of tissue or air space within the fetal lung, which is consistent with the lack of changes in lung growth that were observed. Prenatal ethanol exposure also does not affect alveolar number.

**Table 2. Lung growth and morphometry in control and ethanol-exposed fetuses**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ethanol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung weight, g</td>
<td>133.3 (17.5)</td>
<td>141.2 (40.4)</td>
</tr>
<tr>
<td>Adjusted lung weight, g/kg body wt</td>
<td>32.8 (4.2)</td>
<td>35.2 (6.6)</td>
</tr>
<tr>
<td>Lung volume, cm³</td>
<td>81.9 (6.9)</td>
<td>85.6 (18.0)</td>
</tr>
<tr>
<td>DNA concentration, mg/g tissue</td>
<td>3.5 (0.9)</td>
<td>3.0 (0.9)</td>
</tr>
<tr>
<td>Total DNA content, mg</td>
<td>461.6 (132.6)</td>
<td>379.6 (42.1)</td>
</tr>
<tr>
<td>Adjusted DNA content, mg/kg body wt</td>
<td>112.2 (25.9)</td>
<td>96.5 (12.4)</td>
</tr>
<tr>
<td>Protein concentration, mg/g tissue</td>
<td>33.0 (8.2)</td>
<td>36.0 (7.2)</td>
</tr>
<tr>
<td>Total protein content, mg</td>
<td>4,355.9 (1040.5)</td>
<td>5,077.6 (1170.5)</td>
</tr>
<tr>
<td>Adjusted protein content, mg/kg body wt</td>
<td>1,115.6 (251.1)</td>
<td>1,189.2 (385.9)</td>
</tr>
<tr>
<td>% Tissue-space</td>
<td>84.2 (2.1)</td>
<td>84.1 (2.7)</td>
</tr>
<tr>
<td>% Air-space</td>
<td>16.0 (1.8)</td>
<td>16.0 (2.6)</td>
</tr>
<tr>
<td>Alveolar number (× 10⁶)</td>
<td>3.0 (0.8)</td>
<td>2.6 (0.8)</td>
</tr>
<tr>
<td>Alveolar diameter, μm</td>
<td>48.3 (4.4)</td>
<td>48.5 (4.9)</td>
</tr>
<tr>
<td>Septal thickness, μm</td>
<td>3.0 (0.7)</td>
<td>3.2 (0.5)</td>
</tr>
</tbody>
</table>

Values are means (SD). No values in ethanol-exposed fetuses were significantly different from control values.
alveolar diameter, as measured by the mean linear intercept method, or septal thickness. Therefore, repeated daily ethanol exposure during the third-trimester equivalent does not appear to alter alveolarization. In agreement with this finding, the deposition of elastin in the fetal lung, which occurs at the tips of the secondary crests and regulates alveolar formation (20, 37), was also not altered by ethanol exposure.

Collagen deposition. Prenatal ethanol exposure increases the expression of COLIA1 in the fetal lung and the deposition of collagen fibers types I and III, which constitute ~90% of the collagens within the lung (10). These findings are similar to studies in adults that have shown that alcohol consumption results in increased procollagen type I expression in the liver, duodenum, and pancreas, resulting in fibrosis in these organs (11, 12, 46). An increase in collagen deposition in the lung is also associated with pulmonary fibrosis, which reduces lung distensibility, which in turn decreases forced expiratory volume and forced vital capacity (10). Therefore, an increase in collagen expression and deposition in the fetal lung in response to prenatal ethanol exposure could be detrimental to postnatal lung function if these alterations become more marked and persist after birth.

The mechanisms by which alcohol consumption increases collagen deposition are unknown, but we speculate that they may involve an upregulation of transforming growth factor-β1 (TGF-β1). Previous studies have shown that acetaldehyde, the primary product of ethanol biotransformation, stimulates procollagen type I gene transcription via an increase in TGF-β1 mRNA levels (5, 11, 46). It is well established that TGF-β1 is not only capable of inducing collagen synthesis (5, 32), but can reduce the rate of collagen degradation, resulting in an accumulation of collagen. For example, collagen is degraded by matrix metalloproteinases (MMPs), and the activity of MMPs is inhibited by tissue inhibitors of MMPs (TIMPs). TGF-β1 can reduce MMP expression and upregulate TIMP production, thereby decreasing collagen degradation (5). It also has been postulated that exposure to ethanol results in a modified cross-linked collagen that is less susceptible to collagenases (5). However, this has not been investigated in the present study, and further studies are required to determine if prenatal ethanol exposure alters TGF-β1 expression and downstream signaling events, which could result in the increase in collagen deposition that occurs within the fetal lung.

Ethanol exposure has been shown to cause oxidative stress in pancreatic stellate cells, as indicated by increased lipid peroxidation (3). This increase in oxidative stress is thought to mediate ethanol-induced stimulation of collagen synthesis, because it can be prevented by vitamin E, an antioxidant (3). In
the present study, MDA levels tended to be increased by prenatal ethanol exposure. Because MDA levels are an indication of lipid peroxidation, and therefore oxidative stress, it is possible that the increased collagen synthesis in the fetal lung in response to prenatal ethanol exposure is partly caused by the generation of free radicals during ethanol biotransformation.

Surfactant protein expression. The mRNA expression of SP-A, -B, and -D in the fetal lungs was reduced to approximately one third of control levels following daily ethanol exposure. Because surfactant proteins are components of surfactant, our data indicate that prenatal ethanol exposure decreases surfactant synthesis. As type-II alveolar epithelial cells synthesize and secrete surfactant, a reduction in surfactant protein expression may be due to a reduction in the number of type-II cells. Although previous studies in adults have shown that ethanol exposure can reduce surfactant synthesis and increase type-II cell apoptosis (7, 8, 28, 29), our study has shown that prenatal ethanol exposure does not appear to affect type-II cell number. Alternatively, the reduction in SP expression that occurs in response to prenatal ethanol exposure may be due to a direct effect of ethanol on surfactant synthesis or a reduction in the number of lamellar bodies per type-II cell (lamellar bodies are organelles within type-II cells that produce and store surfactant). The mechanisms involved in the reduction in SP expression are unknown but may be due to oxidative stress and decreased glutathione availability (7, 8, 28, 29).

Surfactant acts to reduce the surface tension at the air-liquid interface within the lung, and this helps to prevent alveolar collapse at end-expiration. Therefore, a reduction in surfactant could increase the risk of respiratory distress syndrome (RDS) at birth in infants that have been exposed to ethanol during pregnancy, especially in those born preterm. It is of interest that chronic ethanol exposure in adults is associated with an increased risk of ARDS (41), which involves decreased surfactant production in the lungs.

A decrease in SP expression may also be a result of a reduction in Clara cell number, because SP-A, -B, and -D are also expressed by Clara cells; Clara cells are nonciliated epithelial cells of the conducting airways and are considered to be the major progenitor cells within the bronchioles (47). Clara cell differentiation is impeded by exposure to compounds that cause lung injury, and it is postulated that these cells have an immunoregulatory role, although their exact role in normal lung development is not well understood (47).

Decreased SP-A and SP-D mRNA levels indicate, in addition to a possible reduction in Clara cell number, that ethanol exposure alters the immune status of the fetus. SP-A and SP-D can bind and opsonize microorganisms and, therefore, can have
an important role in the lung’s primary defense against inhaled pathogens, as well as being an important component of the innate immune response of the lungs. Our findings are in agreement with a recent study in sheep that showed that prenatal ethanol exposure decreases SP-A mRNA and protein levels (36). That study also demonstrated that prenatal ethanol exposure inhibits stimulated tracheal ciliary beat frequency, which is a protective mechanism required to remove foreign particles from the lungs (36). These findings are consistent with other studies that show that ethanol has an overall immunosuppressive effect (17, 22–24, 27). Previous studies in fetal sheep have demonstrated a relationship between infection/inflammation and SP expression; prenatal endotoxin exposure is associated with an increase in SP expression (42, 43). Therefore, we speculate that a decrease in SP expression, as observed in the present study, may be associated with an immunosuppressed state in the fetal lung.

Lung inflammatory status. We have shown that repeated ethanol exposure during the last third of gestation reduces lung proinflammatory cytokine expression, particularly the expression of IL-1β and IL-8, which was reduced by ~90% compared with control levels. A reduction in proinflammatory cytokines in response to alcohol consumption has also been demonstrated in adults (17), and it is thought that, under these circumstances, the lungs would be less able to mount an effective immune response when exposed to pathogens (17). Fetal ethanol exposure has also been shown to decrease macrophage number and function (24), again indicating that ethanol exposure has a suppressive effect on the innate immune system. The long-term effects of a severe reduction in the basal level of proinflammatory cytokines in the lung following fetal ethanol exposure, however, remain to be determined.

Ethanol exposure is known to increase the risk of infection in both adults and neonates (22), and previous studies have shown that maternal alcohol consumption increases the risk of early-onset sepsis in very low birth weight newborns (23). Ethanol exposure can also increase the risk of preterm birth, and preterm infants are more susceptible to respiratory syncytial virus infections (49). These findings indicate that prenatal ethanol exposure is associated with an increased susceptibility to infection in early postnatal life and this could be due, in part, to a reduction in basal proinflammatory cytokine levels.

Conclusions. In conclusion, prenatal ethanol exposure during the third-trimester equivalent increases collagen expression and deposition and decreases SP and proinflammatory cytokine gene expression in the fetal lung. If these changes persist to birth, they could contribute to an increased risk of RDS in the neonate, reduced lung function postnatally, and/or an increased...
susceptibility to neonatal lung infection. Additional studies investigating the effects of fetal alcohol exposure on postnatal respiratory health outcomes in humans are required.

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