Alcohol exposure during late ovine gestation alters fetal liver iron homeostasis without apparent dysmorphism

Foula Sozo,1 Anna M. Dick,1 Jonathan G. Bensley,1 Kelly Kenna,2 James F. Brien,3 Richard Harding,1 and Robert De Matteo1

1Department of Anatomy and Developmental Biology, Monash University, Melbourne, Victoria, Australia; 2Department of Physiology, Monash University, Melbourne, Victoria, Australia; and 3Department of Biomedical and Molecular Sciences, Queen’s University, Kingston, Ontario, Canada

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Sozo F, Dick AM, Bensley JG, Kenna K, Brien JF, Harding R, De Matteo R. Alcohol exposure during late ovine gestation alters fetal liver iron homeostasis without apparent dysmorphism. Am J Physiol Regul Integr Comp Physiol 304: R1121–R1129, 2013.—High levels of alcohol (ethanol) exposure during fetal life can affect liver development and can increase susceptibility to infection after birth. Our aim was to determine the effects of a moderate level of ethanol exposure in late gestation on the morphology, cell proliferation and apoptosis, perivascular collagen deposition, and interleukin (IL)-1β, IL-6, or IL-8 mRNA levels. However, ethanol exposure led to significant decreases in hepatic content of ferric iron and gene expression of the iron-regulating hormone hepcidin and tumor necrosis factor (TNF)-α (all \(P < 0.05\)). In the placenta, there was no difference in transferrin receptor, divalent metal transporter 1, and ferritin mRNA levels; however, ferroportin mRNA levels were increased in ethanol-exposed animals (\(P < 0.05\)), and ferroportin protein tended to be increased (\(P = 0.054\)). Plasma iron concentration was not different between control and ethanol-exposed groups; control fetuses had significantly higher iron concentrations than their mothers, whereas maternal and fetal iron concentrations were similar in ethanol-exposed animals. We conclude that daily ethanol exposure during the third-trimester-equivalent in sheep does not alter fetal liver morphology; however, decreased fetal liver ferric iron content and altered hepcidin and ferroportin gene expression indicate that iron homeostasis is altered.

Clinical and epidemiological data indicate that alcohol consumption during pregnancy remains a substantial public-health problem in countries in which alcohol consumption is a major lifestyle factor (25). For example, in the United States, it has been documented that 30% of women reported at least some alcohol consumption during pregnancy, and 8.3% reported consuming 4 or more alcoholic drinks on one occasion (“binge drinking”) at least once during pregnancy (12). In Denmark, ~25% of more than 85,000 pregnant women surveyed reported at least one episode of “binge drinking” during pregnancy (48). Chronic alcohol consumption during pregnancy can result in fetal alcohol spectrum disorders (FASD), and it is estimated that FASD occurs in as many as 1 in 100 live births (41), although the prevalence could be as high as 2–5% (32).

The brain is a principal target organ of ethanol teratogenicity, and central nervous system injury appears to be the most common and most serious manifestation of FASD (9, 25). However, other organ systems are also susceptible to the teratogenic actions of gestational ethanol exposure, as demonstrated by recent studies on the heart (18, 33), lungs (31, 47, 50), and kidneys (19, 45). Like the adult liver, the developing liver is known to be affected by alcohol exposure (25). Liver fibrosis, fatty degeneration, and abnormal liver function tests have been documented in children with fetal alcohol syndrome (FAS), the most severe type of FASD (22). Furthermore, a recent review of 15 FASD cases, ranging in age from newborn to 8-yr-old, found that hyperbilirubinemia was the most common manifestation of liver injury, occurring in eight subjects, followed by fibrosis/cirrhosis in four subjects and hepatomegaly in three (24). Studies in rats have shown an increase in hepatic cellular fat deposition and decreased liver function in fetuses exposed to ethanol in late gestation (1). In 2-wk-old rats, maternal ethanol intake throughout pregnancy resulted in decreased liver weight, relative to body weight; although liver histology and polyamine synthesis were not affected, hepatic DNA synthesis was decreased (35). Chronic oral administration of low-dose or high-dose ethanol (1 or 5 g ethanol/kg body wt/day) to pregnant rats throughout gestation produced liver injury in the term fetus, manifesting as decreased liver weight relative to body weight, degeneration of the cytoplasm of hepatocytes, increased number of megakaryocytes, and increased tenascin and type IV collagen content in the liver, which appeared to be dose-dependent (30).

The fetal liver plays a major role in prenatal hematopoiesis and lymphopoiesis. Studies have shown that maternal ethanol consumption in rodents substantially decreases pre-B cells and pre-T cells in the fetal liver (43), and the maturation of B cells in the liver in late gestation (3). These changes may decrease the ability of the fetal hematopoietic system to seed the immune system, and may be responsible for the increased risk of infection observed in infants and children exposed to alcohol during fetal life (14, 15).

Our recent research program has focused on the effects of fetal ethanol exposure during the third-trimester-equivalent on various organ systems. This research has utilized time-dated pregnant sheep, which is an established, reliable animal model of maternal ethanol consumption during human pregnancy (10, 46). Our studies have determined that repeated, daily maternal administration of ethanol, equivalent to three standard drinks in...
1 h, produces fetotoxicity involving the kidneys (19), lungs (47), heart (18), and small-resistance arteries (38), and it alters blood gas status (29). The objective of the present study was to test the hypothesis that repeated, daily maternal administration of moderate-dose ethanol during the third-trimester-equivalent of ovine pregnancy produces liver injury in the late-gestation fetus.

METHODS

Ethical approval. All animal procedures were approved by the Monash University Animal Ethics Committee. The treatment and care of animals conformed to the National Health and Medical Research Council of Australia’s Code of Practice for the Care and Use of Animals for Scientific Purposes.

Experimental procedures. Pregnant Border-Leicester × Merino ewes (Ovis aries) underwent aseptic surgery at 91 days of gestational age (DG; term ~ 147 DG) for the chronic insertion of catheters into a maternal carotid artery and jugular vein for blood sampling and ethanol or saline infusion, respectively (47). Anesthesia was induced by a maternal injection of thiopental sodium (1 g iv) and was maintained by inhalation of 1–2% halothane in O2-N2O (50:50 vol/vol). Starting at 95 DG, the ewes were infused intravenously, for 1 h each day, with either 0.75 g ethanol/kg maternal body wt (n = 8) or an equivalent volume of saline (n = 7) until 124 DG; there was one set of twins in each of the control and ethanol-exposure groups. At 126 DG, the ewes underwent further surgery for the implantation of a fetal brachial artery catheter to allow for sampling of fetal blood to assess well-being and to measure plasma ethanol, iron, and zinc concentrations. The daily maternal infusion of ethanol or saline recommenced on 128 DG and concluded on 133 DG. As ethanol readily distributes across the placenta to enter the fetal circulation, fetal and maternal plasma ethanol concentrations were similar; the fetus was exposed to ethanol only via the maternal circulation. The mean maximal ethanol concentration in plasma of exposed ewes and fetuses was 0.12 and 0.11 g/dl, respectively, at 1 h after the infusion onset and had returned to baseline values 7 h after the end of the 1-h infusion; ethanol was undetectable in control ewes or fetuses (47).

Pregnant ewes and fetuses (five males and three females in the control group; five males and four females in the ethanol-exposure group) were humanely killed at 134 DG, 24 h after the last ethanol infusion, by an overdose of pentobarbital sodium (130 mg/kg iv), administered to the ewe. At the time of tissue collection, plasma ethanol concentrations would have been 0 g/dl. At necropsy, the fetus was removed and weighed, and the liver was collected and weighed. Sections of the left and right lobes of the fetal liver were immersion-fixed for 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4) overnight. Sections were then processed and paraffin-embedded for light microscopy analysis. Separate sections of the liver (left and right lobes) were snap-frozen in liquid nitrogen and stored at −80°C for molecular and biochemical analyses; the same lobe of the liver was used for each analysis. Sections of frozen liver were also embedded in optimal cutting temperature (OCT) compound (Tissue-Tek; Sakura) for analysis of lipid deposition.

Histological analysis. Paraffin-embedded tissue from each fetal liver (two sections from the left lobe and two from the right) was sectioned at a thickness of 5 μm. Sections were stained with hematoxylin and eosin to qualitatively assess general morphology, Picrosirius Red to assess collagen deposition, or Perls’ stain to assess ferric iron deposition.

All measurements were performed blinded by the same observer (A. M. Dick) using coded slides. Sections were examined using light microscopy, and digital images were acquired using a digital camera (SPOT Insight 4Meg Fire Wire Color Mosaic 14.2; Diagnostic Instruments) connected to image analysis software (ImagePro Plus, Media Cybernetics).

Collagen deposition. Collagen deposition in the liver was predominately perivascular; therefore, only perivascular collagen was assessed. Analyses were performed at a final magnification of ×200. Five vessels per section were analyzed, and only vessels that appeared in a near-circular cross section were used. In each vessel, the internal perimeter and area of perivascular collagen were measured. The internal perimeter was used as an index of vessel size, and perivascular collagen deposition was normalized by relating it to the internal perimeter of the vessel. On the basis of the internal vascular perimeter, the diameters of analyzed vessels were 169.3 ± 5.9 μm (range: 35.7–648.5 μm). The relative area of perivascular collagen was averaged for each animal and then averaged for each experimental group.

Ferric iron deposition. Analyses were performed at a final magnification of ×200. Images of entire sections were captured using the dotSlide 2.1 imaging system (Olympus) and imported as image stacks to the image analysis program Meta Morph 7.7 (Molecular Devices). The area of ferric iron deposition was automatically measured by the analysis program (based on color using set thresholds) and expressed as a percentage of the area of tissue for each section. The percentage area of tissue stained for ferric iron was averaged for each animal and then averaged for each experimental group.

Lipid deposition. OCT-embedded tissue was sectioned using a cryostat at a thickness of 7 μm. Sections were stained with Oil Red O to qualitatively assess lipid deposition.

Immunohistochemical analysis. Paraffin-embedded sections (5 μm) were immunohistochemically stained with an antibody against either the Ki67 antigen to assess cellular proliferation or cleaved caspase-3 to assess cellular apoptosis.

Sections were incubated at 60°C for ~2 h, deparaffinized in xylene, rehydrated in graded ethanol washes, and washed in PBS. For Ki67, tissue sections were boiled for 20 min in a microwave oven (on high power) in an antigen retrieval solution (Envision Flex target retrieval solution high pH; DakoCytomation). Endogenous peroxidases were blocked using CasBlock (Invironit) prior to a 90-min incubation with a mouse anti-human Ki67 monoclonal antibody (1:100; DakoCytomation). For caspase-3, tissue sections were boiled three times in a microwave oven for 5 min each in 0.01 M citric acid (pH 6.0). Endogenous peroxidases were blocked using CasBlock (Invitrogen), and sections were incubated for 2 h at 4°C with a rabbit anti-mouse/human caspase-3 antibody (1:1,000; R&D Systems). To visualize Ki67 and caspase-3 positively stained nuclei, an EnVision + Dual Link System-HRP (DAB+) immunohistochemistry kit (DakoCytomation) was used. Sections were counterstained with hematoxylin.

Specificity of immunostaining was confirmed by omission of the primary antibody.

Analyses were performed at a final magnification of ×200. Five nonoverlapping fields of view per section were analyzed. The area of tissue containing proliferating cells was measured and expressed as a percentage of the total area of tissue for each field of view. The percentage of tissue containing proliferating cells was averaged for each animal and then averaged for each experimental group. For caspase-3 immunohistochemistry, the number of positively stained cells and the total number of cells were counted for each field of view. For each animal, the number of positively stained cells was expressed as a percentage of the total number of cells counted. This was then averaged for each experimental group.

Gene expression analysis. Relative mRNA levels of ferroportin, hepcidin, interleukin (IL)-18, IL-6, IL-8, and tumor necrosis factor (TNF)-α were measured in frozen liver tissue samples using quantitative real-time polymerase chain reaction (qPCR), as previously described (47). Relative ferroportin, transferrin receptor (TfR1), divalent metal transporter 1 (DMT1), and ferretin mRNA levels were measured in frozen placenta samples collected at necropsy; samples likely contained portions of both the maternal and fetal placenta. Briefly, total RNA was extracted (RNasey Midi kit; Qiagen), DNase-treated (RNase-free DNase kit; Qiagen) and reverse transcribed into...
cDNA (M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant kit; Promega). qPCR was performed using a SYBR Green detection method (Platinum SYBR Green qPCR SuperMix-UDG; Invitrogen Life Technologies) and a Stratagene MX3000P qPCR machine (Agilent Technologies), using reaction conditions previously described (47). Primer sequences, cDNA concentrations, and annealing temperatures used for the amplification of each gene of interest are shown in Table 1. Dissociation curves were performed following the amplification of each gene 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 qPCR experiment. The relative mRNA levels of each gene for each animal were normalized to the mRNA levels of the housekeeping gene ribosomal protein S29 (RPS29) for that animal and analyzed using the ΔΔCt (cycle threshold) method. For each gene, values were expressed relative to the mean mRNA level of the control fetuses.

**Western blot analysis.** Total protein was extracted from placental tissue by homogenization in RIPA buffer [1% Igepal, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM EDTA, 1 × “mini” complete protease inhibitor tablet per 10 ml (Roche Diagnostics)]. The tissue was retained, and protein concentrations were determined using the BCA protein assay (Pierce). The proteins were separated by SDS-PAGE (4–20% gel) were separated by SDS-PAGE (12%) under reducing conditions, and then transferred onto PVDF membranes (Millipore). Membranes were incubated for 1 h in block buffer (5% BSA in TBS containing 0.1% Tween 20; TBST). After blocking, membranes were incubated with rabbit anti-mouse ferroportin polyclonal antibody (1:5,000; MTP11-A, Alpha Diagnostics International) overnight at 4°C. After being washed with TBST, membranes were incubated for 1 h with secondary antibody (HRP-linked donkey anti-rabbit IgG, 1:10,000; GE Healthcare). Membranes were then washed with TBST, and immunoreactive bands detected using Luminata Crescendo HRP substrate (Millipore). To standardize protein loading, membranes were also incubated with rabbit anti-actin polyclonal antibody (1:3,000, A2066; Sigma-Aldrich). The signal was detected using X-ray film, and densitometry analysis was performed using ImageQuant TL analysis software (GE Healthcare).

**Mineral analysis.** Tissue concentrations of iron, copper, and zinc were measured in frozen fetal liver samples by the National Measurement Institute (Melbourne, Australia) using inductively coupled plasma-atomic emission spectrometry (U.S. Environmental Protection Agency 2003, Methods 6020A and 6010B).

Plasma was collected from ewes and fetuses from 131–133 DG at 0 h (just prior to the daily 1-h ethanol or saline infusion) and at 4, 10, and 23 h postinfusion. The concentration of zinc in maternal and fetal plasma samples was measured using an automated enzymatic rate method (Beckman Coulter Synchron DxC800). Briefly, zinc was chelated by 5-Br-PAPS 2-(5-bromo-2-pyridylazo)-5-[N-propyl-N-(3-sulfopropyl)aminol]phenol, and the complex was measured at a wavelength of 540 nm. The mean value for each animal at each time point (0, 4, 10, 23 h) from 131–133DG was used for group comparisons.

Plasma iron concentration in ewes and fetuses was measured using a standard commercial assay (Beckman Coulter Synchron DxC800). Briefly, iron released from transferrin by acetic acid was reduced to the ferrous state by hydroxylamine and thioglycolate. The ferrous ion was immediately complexed with the Ferrozine Iron Reagent (Beckman Coulter), and the change in absorbance was measured at a wavelength of 560 nm.

**Statistical analysis.** Data are presented as means ± SE. Student’s unpaired t-test was used to compare the mean values from control and ethanol-exposed fetuses. Two-way repeated-measures ANOVA was used to compare plasma zinc concentration in control ewes and fetuses and in ethanol-exposed ewes and fetuses from the start of the infusion (0 h) to 23 h postinfusion. Two-way ANOVA was used to compare plasma iron concentration with treatment (control vs. ethanol) and generation (ewe vs. fetus) as factors. If the ANOVA was significant, post hoc analysis was conducted. Statistical analysis was performed using IBM SPSS Statistics (version 18 for Mac). Regression analysis was performed using GraphPad Prism (version 5.0 for Mac). A P value of <0.05 was considered to be statistically significant.

### RESULTS

**Liver weight.** Absolute liver weight was not different between groups (control: 104.6 ± 8.8 g; ethanol: 103.7 ± 13.3 g). There was also no significant difference between groups in liver weight adjusted for body weight (control, 25.6 ± 1.9 vs. ethanol, 26.5 ± 2.8 g/kg); body weight was not different between control (4.1 ± 0.2 kg) and ethanol-exposed (3.8 ± 0.2 kg) fetuses.

**Liver morphology.** In assessing general liver morphology, there was no evidence of overt tissue injury, fibrosis, or gross

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Nucleotide sequences of forward (F) and reverse (R) primers used to amplify a region of each ovine gene of interest with the listed GenBank accession number. Annealing temperatures (Tm) and starting cDNA and primer concentrations used for amplification are shown.
morphological changes in control and ethanol-exposed fetuses. Lipid deposits were not observed in any fetal liver sample from either treatment group; lipids were evident in the positive control skin tissue section.

Hepatic perivascular collagen deposition. The area of perivascular collagen deposition relative to the internal perimeter of the blood vessel was not significantly different between treatment groups (control, 5.7 ± 0.4 vs. ethanol, 5.9 ± 0.4 \( \mu m^2/\mu m \)).

Hepatic cell turnover. The majority of proliferating cells in fetal liver tissue were present in clusters, likely to be hematopoietic stem cell clusters. There was no significant difference in the percentage area of liver tissue containing proliferating cells between control (4.1 ± 0.9%) and ethanol-exposed (6.1 ± 1.2%) fetuses. Similarly, there was no significant difference in the percentage of hepatic cells undergoing apoptosis between groups (control, 50.3 ± 2.4 vs. ethanol, 45.2 ± 3.6%).

Hepatic iron status. The percentage area of liver tissue containing ferric iron was significantly lower in ethanol-treated fetuses (0.5 ± 0.2%) compared with controls (2.9 ± 0.9%; \( P = 0.014 \); Fig. 1). However, the concentration of total iron in liver tissue was not significantly different between control fetuses (406.5 ± 104.8 mg/kg liver wt) and ethanol-exposed fetuses (228.6 ± 84.2 mg/kg liver wt; \( P = 0.207 \)). The relative gene expression of the iron-regulating hormone hepcidin was significantly lower in ethanol-exposed liver (0.2 ± 0.1) compared with control liver (1.0 ± 0.2; \( P = 0.011 \); Fig. 2A). The relative gene expression of the iron transporter ferroportin in the liver was similar in control fetuses (1.0 ± 0.1) and ethanol-exposed fetuses (1.1 ± 0.1). Hepatic ferroportin mRNA levels were significantly negatively correlated with hepatic hepcidin mRNA levels in control fetuses only (\( R^2 = 0.632; P = 0.018 \); Fig. 2B), although the slopes of the regression lines were not different between control and ethanol-exposed fetuses (\( P = 0.594 \)).

Systemic iron status. Plasma iron concentration in ethanol-exposed ewes (27.1 ± 2.5 \( \mu mol/l \)) was not different to that in control ewes (23.6 ± 0.7 \( \mu mol/l \)). Similarly, plasma iron concentration in ethanol-exposed fetuses (23.6 ± 4.4 \( \mu mol/l \)) was not significantly different to that in control fetuses (33.2 ± 2.2 \( \mu mol/l \)). However, there was a significant interaction between treatment and generation factors (\( PTG = 0.033 \)), such that control fetuses had a significantly higher iron concentration than their mothers (\( P = 0.009 \)); this pattern was not evident in ethanol-exposed fetuses (Fig. 3A). Fetal plasma iron concentration tended to be positively correlated with hepatic hepcidin mRNA levels in ethanol-exposed fetuses only (\( R^2 = 0.606; P = 0.068 \); Fig. 3B). The slopes of the regression lines also tended to be different between control and ethanol-exposed fetuses (\( P = 0.051 \)), such that for a specific reduction in hepcidin levels, plasma iron concentration tended to be decreased more in ethanol-exposed fetuses compared with controls.

Regulation of iron transport across the placenta. Relative mRNA levels of ferroportin in the placenta were significantly increased in ethanol-exposed animals (1.4 ± 0.0) compared with controls (1.0 ± 0.1; \( P = 0.001 \); Fig. 4A). Ferroportin protein levels tended to be increased in ethanol-exposed ani-

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Fig. 1. Hepatic ferric iron deposition. Representative photomicrographs of ferric iron deposition (blue) in a control fetus (A) and an ethanol-exposed fetus (B). Scale bar is 100 \( \mu m \). The area of liver tissue containing ferric iron (C) in control fetuses (●) and ethanol-exposed fetuses (○). Data points are from individual fetuses and means ± SE are shown. *\( P < 0.05 \).
ethanol: 0.9 ± 0.1) were not different between groups. However, relative TNF-α mRNA levels were significantly lower in ethanol-exposed fetal livers (0.5 ± 0.1) compared with controls (1.0 ± 0.1; \( P = 0.016 \); Fig. 5).

**DISCUSSION**

The present study demonstrates that daily exposure to ethanol during the third-trimester-equivalent of ovine pregnancy decreases fetal hepatic content of ferric iron and relative mRNA levels of hepcidin, an iron-regulating hormone, and TNF-α, a proinflammatory cytokine. Ethanol exposure also increases the relative mRNA levels and protein levels of the iron transporter ferroportin (control: 1.0 ± 0.1; ethanol: 1.3 ± 0.2; Fig. 4B); however, this did not quite reach statistical significance (\( P = 0.054 \)). Ethanol exposure did not alter the relative mRNA levels of TfR1 (control: 1.0 ± 0.2; ethanol: 1.0 ± 0.3), DMT1 (control: 1.0 ± 0.1; ethanol: 1.3 ± 0.1) and ferritin (control: 1.0 ± 0.1; ethanol: 1.0 ± 0.2) in the placenta.

**Mineral concentrations.** The concentrations of zinc (control: 110.8 ± 15.8 mg/kg liver wt; ethanol: 96.3 ± 22.7 mg/kg liver wt) and copper (control: 37.6 ± 3.1 mg/kg liver wt; ethanol: 41.4 ± 5.5 mg/kg liver wt) in fetal liver tissue were not different between groups. Similarly, the concentrations of zinc in both maternal and fetal plasma were not different between treatment groups (control maternal: 9.8 ± 0.5 µmol/l; ethanol maternal: 9.5 ± 0.5 µmol/l; control fetal: 10.3 ± 0.9 µmol/l; ethanol fetal: 10.5 ± 1.2 µmol/l).

**Hepatic proinflammatory cytokine gene expression.** The relative gene expression of hepatic IL-1β was not significantly different between control (1.0 ± 0.1) and ethanol-exposed (0.8 ± 0.2) fetuses. Similarly, the relative mRNA levels of IL-6 (control: 1.0 ± 0.3; ethanol: 0.6 ± 0.1) and IL-8 (control: 1.0 ± 0.2; maternal: 9.5/11006)_\textit{AJP-Regul Integr Comp Physiol} • doi:10.1152/ajpregu.00479.2012 • www.ajpregu.org
ferroportin in the placenta and counteracts the greater plasma iron concentration observed in control fetuses relative to ewes. There was no evidence of ethanol-induced changes in fetal liver weight, liver morphology, hepatic cell proliferation and apoptosis, fat deposition, perivascular collagen deposition, concentrations of total iron, copper and zinc, or IL-1β, IL-6, or IL-8 relative mRNA levels in the fetal liver. These findings indicate that iron homeostasis may be altered by repeated ethanol exposure in the late-gestational-age fetus in the apparent absence of hepatic dysmorphology.

Liver growth and morphology. Previous studies in sheep in the third-trimester-equivalent (8) suggest that ethanol is primarily metabolized by alcohol dehydrogenase in the maternal liver and acetaldehyde, the primary metabolite of ethanol, is metabolized by aldehyde dehydrogenase by either the maternal or fetal liver (and placenta), depending on the concentration. As ethanol and acetaldehyde cross the placenta and the fetal liver has limited alcohol-metabolizing capacity, the fetal liver is likely to be sensitive to injury induced by alcohol.

We administered ethanol at the same time each day during the third-trimester-equivalent of gestation, sufficient to produce maximal plasma ethanol concentrations in the fetus of 0.11 g/dl; this equates to 3.8 U.S. standard alcoholic drinks in 1 h. The lack of effect of this ethanol regimen on fetal body weight (47) and liver weight in late gestation is consistent with findings in the offspring of pregnant rats that consumed an ethanol-containing diet only during the second half of gestation (35). In contrast, chronic oral administration of ethanol (1 g/kg maternal body wt/day) throughout pregnancy of rats decreased fetal liver weight relative to body weight at term (30), as did volitional maternal consumption of ethanol throughout pregnancy in offspring at 2 wk of age (35). Studies in sheep have also reported decreased liver weight in fetuses exposed to ethanol prior to and throughout pregnancy (40). Our observed lack of effect of repeated ethanol exposure during the third-trimester-equivalent on fetal liver morphology, fat deposition, cell turnover, and perivascular collagen deposition is in contrast to histological changes in the fetal rat liver following ethanol exposure throughout pregnancy (30). Structural effects in the fetal rat liver included degeneration of the cytoplasm of hepatocytes, increased number of megakaryocytes, and increased tenasin and type IV collagen (30). Thus, it is likely that repeated ethanol exposure throughout most of gestation might be necessary to produce overt morphological changes in liver development in the fetus and young postnatal offspring. Our histological results in the liver are consistent with our previous studies of the brain of the same fetuses, where no major structural alterations were observed in the white matter (29).

Hepatic inflammation. Our regimen of daily, moderate-dose ethanol exposure during the third-trimester-equivalent of ovine pregnancy produced selective molecular changes in the fetal liver. These involved decreased hepatic expression of the proinflammatory cytokine TNF-α, with no effect on IL-1β, IL-6, or IL-8. This decrease in TNF-α gene expression alone is in contrast to the decrease of both IL-1β and IL-8 relative mRNA levels, but not of IL-6 or TNF-α, that followed repeated ethanol exposure in the lungs of the same fetuses (47), and the ethanol-induced increase in TNF-α, but not of IL-1β, IL-6, or IL-8 in the placenta (29). It would appear that the effect of repeated prenatal ethanol exposure during late gestation on the expression of specific proinflammatory cytokine genes is tissue-selective, at least in the ovine fetus. Previous studies have reported that ethanol exposure alters B-cell maturation in the fetal liver (3, 43) and causes more severe liver infection in adult rodents when exposed to Listeria monocytogenes (39) or Salmonella typhimurium (44). These findings are consistent with the observed immunosuppressive effects of ethanol in other organs in adults (11, 20), as well as in prenatally exposed offspring (14–16, 21, 26, 27, 34, 51). As the fetal liver plays a major role in prenatal hematopoiesis and lymphopoiesis, further studies are required to determine whether a compromise in postnatal immune function is due to decreased ability of the fetal hematopoietic system to seed the immune system.

Iron homeostasis. We found that daily ethanol exposure decreased fetal hepatic content of ferric iron and relative mRNA levels of hepcidin. Decreased hepatic gene expression of hepcidin has also been reported in a mouse model of FAS following exposure to moderate doses of ethanol in early gestation (28). The precise mechanism by which ethanol alters hepcidin mRNA levels is unknown, although previous studies have demonstrated that proinflammatory cytokines, in particular, IL-6, can regulate hepcidin gene expression (4). Hepcidin expression is also regulated by the amount of iron in the liver, such that if iron levels decrease, hepcidin levels decrease (37). Indeed, ferric iron deposition in ethanol-exposed fetal livers
was decreased in our study, and mean total iron concentration was \( \sim 45\% \) lower compared with controls, although this difference was not statistically significant, likely because of the high variability observed between individual animals. While not statistically significant, these findings may be biologically significant. The technique we used for measuring ferric iron in the liver cannot detect bound iron (ferritin, transferrin, and hemoglobin). In the ethanol-exposed fetal liver, more iron may have been bound to protein, but this would only have been detected when we undertook the total iron concentration measurement. As hepcidin itself is an iron-regulating hormone, these findings may be indicative of altered iron homeostasis. Hepcidin is also down-regulated in alcoholic liver disease in adult rats, but this is related to an increase in tissue iron due to excessive absorption of iron from the gut (4). In the present study, ethanol exposure did not cause a significant change in maternal plasma iron concentration, and in our previous studies, we reported no effect of ethanol exposure on hemoglobin or hematocrit levels or blood oxygenation (29). In the ewes, we measured only plasma iron concentration (and blood gas status in our previous study), and this was not affected by ethanol exposure; therefore, it is unlikely that ethanol disrupted iron regulation in the maternal liver. However, we cannot exclude the possibility of altered maternal iron status, as plasma iron concentration is quite stable, even in the presence of markedly reduced iron stores. This question should be addressed in future studies.

In contrast to adults, in whom iron is obtained from the diet, fetuses obtain iron from the mother by transfer across the placenta. It is known that the fetus regulates mobilization of iron from its mother depending on its demand for iron, which increases during late gestation, resulting in higher iron concentration in the fetus than in the mother (7). In ethanol-exposed fetuses plasma iron concentration was similar to those of their mothers, suggesting disrupted placental iron transfer. It is well established that hepcidin regulates iron transport and iron availability by binding to ferroportin, resulting in the internalization of ferroportin from the cell membrane and subsequently its degradation; ferroportin acts as a channel for the transport of iron out of the cell and into the circulation (36). The close relationship between hepcidin and ferroportin levels was evident in our study, where we found a significant inverse linear correlation between hepatic hepcidin mRNA levels and hepatic ferroportin mRNA levels in control fetuses; ferroportin levels increased as hepcidin levels decreased. However, despite a reduction in hepcidin gene expression in the fetal liver in response to ethanol exposure, ferroportin gene expression was not altered.

Fetal hepatic hepcidin can also act on the placenta to regulate iron transport (13). In the placentas of ethanol-exposed animals we found a significant increase in ferroportin mRNA levels and a strong tendency for ferroportin protein levels to be increased. The change in ferroportin levels in the placenta would be expected to favor transport of iron from mother to fetus; however, it appears that the change in ferroportin levels were not sufficient to increase the transfer of iron from the circulation of the ewe to the fetus, as there was no difference in iron concentration between them. As our findings suggested that placental transport of iron may be altered by ethanol exposure, we investigated other factors involved in this process. Iron in the maternal circulation is normally bound to transferrin and would be expected to bind to the transferrin receptor (TfR1) in the microvillus membrane surface in the placenta (7). TfR1 mRNA levels were not altered in the placenta by ethanol exposure, suggesting that the transport of iron from the placenta was not disrupted. The iron-transferrin-TfR1 complex is internalized via clathrin-coated vesicles and moves into the endosome, resulting in the dissociation of iron from the transferrin-TfR1 complex (7). Divalent metal transporter-1 (DMT1) acts as a channel to transport the iron (now \( \text{Fe}^{2+} \)) out of the endosome and to the cell cytoplasm, where it then exits the cell and enters the fetal side of the placenta via ferroportin, which is located in the syncytiotrophoblast basal membrane (7); although DMT1 mRNA levels were not altered,
ferroportin levels were increased. Zyklopen then oxidizes the iron to ferric iron (Fe³⁺), where it is either stored as ferritin in the stroma or binds to fetal transferrin to enter the fetal circulation (7). As we were unable to measure zyklopen levels, we cannot comment on whether ethanol exposure results in altered iron oxidation. However, ferritin mRNA levels were not altered in the placenta by ethanol exposure, suggesting that if iron were not being transported to the fetus, it was likely not being stored in excess in the placenta.

It remains to be determined whether there is an effect of repeated maternal ethanol administration on hematopoiesis in the liver of the late-gestational-age ovine fetus and whether there is a relationship between the apparently altered fetal hepatic iron homeostasis during the third-trimester-equivalent of pregnancy and the occurrence of hyperbilirubinemia observed in individuals with FASD (24).

Zinc concentration. Ethanol exposure during early-midgestation has previously been linked to decreased fetal zinc concentration (5, 6, 17, 49). Acute ethanol exposure has been shown to induce metallothionein in the maternal liver, which binds to zinc and, hence, decreases plasma zinc concentration (6). Because the fetus obtains zinc via placental transfer from the mother, this indicates that zinc distribution may be impaired following ethanol exposure, possibly in a metallothionein-dependent manner (5, 6). In our study, however, plasma zinc concentrations in ewes and fetuses, and fetal liver zinc concentration were not altered by ethanol exposure. Decreased plasma zinc concentration can be an indication of fetal nutritional deficits and has been linked to fetal growth restriction (17, 42). Additionally, zinc is an essential component of more than 300 enzymes that are used to provide cell structural stability, regulation of transcription and translation processes, and decreased oxidative stress levels (52). Therefore, our finding of similar zinc concentrations between control and ethanol-exposed fetuses is consistent with the absence of altered somatic and liver growth and liver morphology; the difference between our findings and previous studies is likely due to the relatively lower dose of ethanol used in our study compared with previous studies, as well as differences in the gestational timing of ethanol exposure. In support of this hypothesis, exposure of pregnant mice to a low blood ethanol concentration throughout gestation does not change plasma zinc concentration and does not result in fetal teratogenicity (P. Coyle, personal communication).

Conclusion. We conclude that daily, moderate-dose ethanol exposure during the third-trimester-equivalent does not alter fetal liver growth or morphology. However, this ethanol exposure regimen decreased fetal liver ferric iron content and hepatic hepcidin gene expression, increased placental ferroportin gene expression, and tended to increase ferroportin protein levels in the placenta. Ethanol exposure also inhibited the normal increase in the circulating iron concentration of the fetus relative to the mother, indicating altered iron homeostasis.

Perspectives and Significance

In summary, exposure to a moderate dose of ethanol during the third-trimester-equivalent appears to alter iron homeostasis in the fetus and may affect immune function, without overt changes in liver morphology. Altered iron homeostasis may affect tissue oxygen delivery and multiple enzyme reactions, including those involved in oxidative stress pathways. Furthermore, as the fetal liver is the major site of hematopoiesis, it will be important in the future to determine whether maturation of blood cells is altered and whether this is responsible for the hyperbilirubinemia and immunosuppressive effects observed in response to ethanol.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


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