Daily ethanol exposure during late ovine pregnancy: physiological effects in the mother and fetus in the apparent absence of overt fetal cerebral dysmorphology

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MATERNAL INGESTION OF ETHANOL (EtOH) in high doses during pregnancy can lead to the fetal alcohol syndrome (FAS) or fetal alcohol spectrum disorders (FASD) in offspring (27). The major debilitating effects of FAS and FASD that have been commonly described are neurocognitive impairments including learning disabilities, visual-spatial deficits, and delayed language and motor development (27). Infants with FAS or FASD are likely to experience lower birth weight due to fetal growth restriction, and this in itself may have long-term adverse effects on health. Recent surveys have shown that many pregnant women consume moderate amounts of EtOH on a daily or near-daily basis, in an amount that may not induce intoxication and apparently does not lead to detectable FAS or FASD (21). Although the effects of high levels of EtOH ingestion producing FAS and FASD are well documented, the effects of daily maternal consumption of moderate doses of EtOH on the fetus are less well understood. It is likely that daily exposure of the fetus to moderate levels of EtOH will produce different developmental effects compared with chronic, daily, high-dose EtOH ingestion or infrequent high-dose binge drinking.

In a recent study we used late gestation pregnant sheep to determine the effects of 1-h maternal EtOH infusions over three successive days (116–118 days of the 145-day gestation) on fetal brain development and fetal growth (10, 14). We chose to focus on the third trimester equivalent because it is a critical period of vulnerability for fetal brain development, especially for migration and differentiation of neurons, synaptogenesis, and development (32). Each daily EtOH infusion raised maternal and fetal plasma EtOH concentrations (PEC) to a maximal value of 0.11 g/dl, the equivalent of a 60–70 kg woman consuming 3–4 USA standard drinks in 1 h (25). Five days after the initial EtOH exposure, there was evidence of injury and altered development of white matter in the subcortical region of the fetal brain, including gliosis, altered glial cell morphology, axonal disruption, and increased apoptosis (10). We also found that this regimen of daily EtOH administration for 3 days led to a reduction in fetal body weight of 19% compared with controls, and reductions in maternal plasma IGF-I and fetal plasma IGF-II concentrations (14). In view of these findings, we questioned whether daily exposure to a similar peak plasma concentration of EtOH over a period greater than 3 days would have more severe effects on fetal growth and brain development or whether fetal adaptation to this more chronic exposure would occur.

In the present study, the period of daily EtOH exposure during ovine pregnancy was increased from 3 days to 39 days, beginning earlier in the third trimester equivalent and continuing until later in gestation. Our objectives were to determine the effects on the fetal brain of daily EtOH exposure that was equivalent to 3.8 USA standard drinks throughout most of the third trimester equivalent of pregnancy; this is equal to 5.6 UK standard drinks, 3.3 Canadian standard drinks, and 4.5 Australian standard drinks. We have also assessed the effects of EtOH...
on maternal and fetal cardiovascular, metabolic, and oxygenation status, because in our previous study, blood samples and cardiovascular parameters were obtained over a limited period following each EtOH exposure (10, 14). Furthermore, there is still some controversy as to the role of fetal hypoxemia in the etiology of EtOH-induced injury to the fetal brain (9, 41). Fetuses were exposed to EtOH on a daily basis from 95 to 133 days of gestation (DGA), a period that spans the developmental period when the fetal forebrain is maturing and white matter tracts are developing (32). The maximal plasma concentrations of EtOH in the mother and fetus were similar to those attained in our previous studies of 3 days of EtOH exposure (10, 14). We hypothesized that overt fetal brain injury and growth restriction following a prolonged period of EtOH exposure would be greater than we observed during three days of a similar degree of EtOH exposure.

MATERIALS AND METHODS

All animal procedures were approved by the Monash University Animal Ethics Committee.

Surgical Procedures

At 92 DGA, date-mated mixed-breed ewes underwent aseptic surgery for implantation of polyvinyl catheters into a maternal jugular vein and carotid artery. After recovery, these catheters were used for intravenous infusions of either EtOH or saline and for sampling maternal arterial blood. At 126 DGA, the ewes underwent further aseptic surgery for implantation of catheters into a fetal brachial artery and the amniotic sac; these were used for sampling of fetal blood and amniotic fluid, respectively. While our preference was for singleton-bearing ewes, at surgery twin fetuses were found to be present in one control and one treated ewe; in these cases, only one of the twins was catheterized. Following each period of surgery, ewes were administered intravenous antibiotics (500 mg ampicillin; Douglas Pharmaceuticals, Australia) for 3 days.

Experimental Protocol

The ewes were randomly assigned to either an EtOH infusion group (n = 8 fetuses) or a control group (n = 8 fetuses). The EtOH group received daily, 1-h infusions of EtOH (iv), starting at ~09.00 h each day, from 95 to 124 DGA and from 128 to 133 DGA; infusions were not performed on 125–127 DGA to avoid the period of fetal surgery. Absolute EtOH was diluted to a 40% solution with saline and was infused to deliver 0.75 g EtOH/kg of maternal body weight. Ewes were weighed every 2 wk so that the EtOH dose could be adjusted for maternal weight gain. This dose was chosen to obtain maximal PEC (∼120 g) to compensate for the additional calories contained in EtOH (7.1 calories/g). Ewes were fed at ~08.30 h each day.

Over 3 days (131–133 DGA), maternal (3 ml) and fetal (3 ml) arterial blood samples were taken immediately before each infusion (time = 0 h) and at 1, 2, 4, 6, 8, 10, and 24 h after the infusion onset; a total of 24 ml of blood was removed from the fetus on each of the 3 days. Red blood cells were reinjected into the fetus if hemoglobin (Hb) concentration dropped below 30%; however, this was only required in one fetus from the EtOH group. PEC was analyzed using the Dade Behring Dimension RxL Clinical Chemistry System (10, 14). Fetal and maternal blood samples were also analyzed (model ABL700; Radiometer, Denmark) to determine arterial partial pressure of oxygen (Pao2), saturation of oxygen (Sao2), partial pressure of carbon dioxide (Paco2), pH, glucose, lactate, Hb, and hematocrit (Hct). Fetal mean arterial pressure (MAP) and heart rate (HR) were recorded continuously (PowerLab8/30; ADInstruments, Australia) from 131 to 133 DGA. Fetal HR was derived from the arterial pressure waveform. The digitized data were subsequently analyzed over 10-min intervals to obtain hourly values. Arterial pressure was adjusted for amniotic pressure.

Ewes and fetuses were killed at 134 DGA (term: 147 DGA) owing to the risk of preterm birth associated with EtOH administration (28). At 134 DGA, the ewe and fetus were killed with pentobarbitonal sodium (130 mg/kg iv) administered intravenously to the ewe. Fetuses were weighed and crown-to-rump length (CRL), thoracic girth, forelimb length, and head length were measured; the fetal ponderal index (body wt ÷ CRL3) was calculated. Major organs were collected and weighed. The fetal brain was removed and placed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4).

Histological Analysis of the Fetal Brain

The cerebrum of each fetus (n = 16) was positioned in a prescribed orientation to ensure uniformity of sectioning angle and was cut into 5-mm coronal blocks (8–10 per animal); one block (5 mm thick) was also taken from each cerebellar vermis. All blocks were embedded in paraffin wax. Six to eight sections (10 μm) were collected from the parietal, temporal, and occipital lobes and cerebellar vermis for analysis.

Qualitative Histological Analysis

A section from each block of the cerebrum and cerebellum was stained with hematoxylin and eosin and examined for the presence of parenchymal or subarachnoid hemorrhages, neuronal necrosis, cystic infarction, or overt white matter injury.

Immunohistochemistry

Paraffin sections from each cerebral lobe were reacted with the following antibodies: rabbit antitgbal fibrillary acidic protein (GFAP; 1:500, code no. 20334; Dako, Glostrup, Denmark) to identify reactive astrocytes; rabbit anti-ionized calcium-binding adapter molecule 1 (IBA-1; 1:1,500, code no. 019–19741; Wako, Richmond, VA) to identify microglia. Sections from the cerebellar vermis were also reacted for GFAP immunoreactivity (IR). To identify myelinating oligodendrocytes rat antiamyelin basic protein (MBP; 1:200; Chemicon International) was used. All sections were incubated in the appropriate secondary antibodies (1:200) for 90 min and reacted using the avidin-biotin peroxidase complex kit (Vector Laboratories, Burlingame, CA). Before incubation with primary antibodies, IBA-1 and MBP sections were pretreated with citrate buffer (pH 6.0) for 7 min in a microwave oven. Sections from each lobe were stained with DeadEnd Colorimetric TUNEL System (Permea, Madison, WI) to identify cell death (apoptotic and necrotic). For each antibody, sections from each treatment group were simultaneously reacted to reduce staining variability. There was no staining when the primary antibodies were omitted.

Quantitative Morphometric Analysis

In the fetal cerebrum. Measurements were made in each fetus on coded slides by an observer blinded to treatment group using an image analysis system (Image-Pro Plus version 6.2; Media Cybernetics, Frederick, MD). In each slide, a field of view refers to an area of 0.2 mm2. Consistent regions of the parietal, temporal, and occipital lobes were examined in each analysis. Each section was projected onto a digitizing tablet, and the following measurements were made. First, cells immunoreactive for GFAP were counted in four randomly selected fields in the gray matter, two fields in subcortical white matter and two fields in deep white matter. A mean value (8 fields for gray matter, 4 for subcortical white matter, and 4 for deep white matter)
Gene Expression Analysis

Relative mRNA levels of the proinflammatory cytokines IL-1β, IL-6, and TNF-α were measured using quantitative real-time PCR (qPCR) in fetal white matter from the parietal lobe that was snap-frozen on the day of necropsy and then stored at −80°C; placental tissue from each animal was also analyzed. Total RNA was extracted using an RNasy kit (Qiagen, Australia), treated with DNase (Qiagen, Australia) 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) by using reaction conditions as previously described (37). Primer sequences, cDNA concentrations, and annealing temperatures used for the amplification of each gene are shown in Table 1. Dissociation curves were performed following the amplification of each gene to ensure that a single PCR product was amplified per primer set. Samples were measured in triplicate with a negative control sample, which did not contain template cDNA, included in each qPCR run. The relative mRNA levels of each gene for each animal were normalized against the mRNA levels of the housekeeping gene ribosomal protein S29 (RPS29) for that animal and were analyzed using the change in cycle threshold method. There was no difference in the expression of RPS29 between treatment groups. Values are presented relative to the mean mRNA level of the control animals for each gene.

Statistical Analysis

Physiological data from each ewe and fetus were averaged for each time point over the 3-day period (i.e., from 131 to 133 DGA); data were not different on each of these days. The mean data from each fetus were then analyzed from the start of the infusion period (time = 0 h) to 10 h postinfusion using a one-way repeated-measures ANOVA. If treatment or interaction terms were significant, the unpaired Student’s t-test was used to determine differences between groups; this test was also used to analyze morphometric, necropsy, neuropathology, and gene expression data. Statistical significance was accepted at P < 0.05. Data are presented as means ± SE.

RESULTS

Plasma EtOH Concentrations

One hour after the start of the EtOH infusions on 131–133 DGA, PEC reached similar maximal values in the ewe (0.117 ± 0.005 g/dl, n = 8) and fetus (0.107 ± 0.006 g/dl, n = 7). PEC then progressively declined in the ewe and fetus, reaching nonmeasurable levels by 8 h after the start of the EtOH infusion (Fig. 1). Fetal and maternal PECs were not significantly different from each other at any time point.

Maternal Physiological Data

Following each daily EtOH infusion, maternal PaO2, (Fig. 2A) and SaO2 (data not shown) were not different between EtOH and control groups. Similarly, maternal PaCO2 following

Table 1. Primer sequences

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<th>Forward</th>
<th>Reverse</th>
<th>Temp</th>
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<tr>
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<td>TNF-α</td>
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Nucleotide sequences for each forward and reverse primer (5’–3’) were used to amplify each gene of interest. Primer sequences were designed based on the nucleotide sequence that corresponds to the listed GenBank accession number. The annealing temperature (Temp) and template cDNA concentrations used for the amplification of each gene are shown.
After EtOH infusions, maternal blood lactate concentration fell below control values, but there was only a nonsignificant trend for an overall treatment effect ($P_{\text{treat}} = 0.089$). However, there was a highly significant interaction between treatment and time ($P_{\text{treat}\times\text{time}} = 0.000$, Fig. 3A); glucose concentrations in EtOH ewes were significantly lower than in the control ewes by 44%, 46%, and 33% at 1, 2, and 4 h, respectively, after the start of the EtOH infusion. Maternal blood lactate concentration increased significantly at the end of the EtOH infusion (1 h) and remained elevated at 2, 4, and 6 h compared with the control group (Fig. 3B). The maximal maternal blood lactate concentration was $1.56 \pm 0.19 \text{ mmol/l}$ and occurred at 4 h after the start of the EtOH infusion.

**Fetal Physiological Data**

The changes in fetal PaO$_2$ and SaO$_2$ after the EtOH infusions were significantly different in the EtOH exposed and control groups (Fig. 4, A and B). Fetal PaO$_2$ slowly fell after the EtOH infusion and at 10 h reached $20.7 \pm 0.5 \text{ mmHg}$, which was significantly (17%) lower than in controls (Fig. 4A). Fetal PaO$_2$ had returned to control values at 24 h after the EtOH infusion. Fetal SaO$_2$ also fell after the EtOH infusion and was, respectively, 16% and 21% lower than in the control group at 8 and 10 h (Fig. 4B); the minimal SaO$_2$ occurred at 10 h, as with fetal PaO$_2$. There was no difference between the EtOH-treated and control groups in fetal PaCO$_2$, or arterial pH (Fig. 4, C and D); however, in both EtOH and control groups, fetal PaCO$_2$ gradually decreased and pH increased over time, with each parameter returning to control values by 24 h.

Baseline blood glucose concentration in fetuses was significantly lower than in ewes, as expected (0.68 ± 0.12 vs. 2.23 ± 0.18 mmol/l, $P < 0.05$). There was no overall treatment effect for fetal blood glucose concentrations, but there was a highly significant difference between groups in the changes with time ($P_{\text{treat}\times\text{time}} = 0.001$, Fig. 3C). There was a near-significant trend for blood glucose concentrations in the EtOH fetuses to be lower than in control fetuses at 1 h ($P = 0.071$) and 2 h ($P = 0.056$) after the start of the infusions. As in the EtOH-treated ewes, blood glucose concentrations progressively increased in EtOH fetuses between 2 and 10 h after the start of the EtOH infusions.

Baseline blood lactate concentration in fetuses was significantly higher than in ewes (1.1 ± 0.1 vs. 0.3 ± 0.0 mmol/l, $P < 0.05$), but there were no differences between treatment groups in either fetuses or ewes. In EtOH-exposed fetuses, lactate concentrations were significantly higher than in control fetuses at 4, 6, 8, and 10 h after the start of the infusions (Fig. 3D). In EtOH fetuses, the maximal lactate concentration of 2.0 ± 0.2 mmol/l, measured at both 8 h and 10 h, was not significantly higher than the maximal value in ewes (1.6 ± 0.2 mmol/l at 4 h).

There were no significant differences between treatment groups in baseline values of fetal Hb, Hct, MAP, or HR, and values were not different between groups after the infusions. Mean values for both groups combined were: Hb, 10.1 ± 0.3 g/dl; Hct, 31.2 ± 0.8%; MAP, 40 ± 2 mmHg; HR, 162 ± 4 beats/min.

**Necropsy Data**

At necropsy (134–135 DGA) the body weight of EtOH fetuses was 3.8 ± 0.3 kg ($n = 8$), which was not different from that of control fetuses (4.1 ± 0.2 kg, $n = 8$, Table 2).
were no differences in CRL, thoracic girth, forelimb length, head length, or ponderal index between EtOH and control fetuses (Table 2). Heart weight relative to body weight tended to be greater in EtOH fetuses ($8.6 \pm 0.7 \text{ vs. } 7.2 \pm 0.5 \text{ g/kg, } P = 0.088$). There were no differences between treatment groups in weights of other organs (Table 2).

Neuropathology

Qualitative Analysis of the Cerebrum and Cerebellum

There was no evidence of cystic lesions, major neuronal necrosis, or overt white matter injury in the cerebrum or cerebellum of any fetus. In three of the eight EtOH fetuses, minor subarachnoid hemorrhages were visible in at least one section from the cerebellum and/or cerebrum; no hemorrhages were seen in control fetuses. In one fetus, hemorrhages were observed in both the cerebrum and cerebellum. An example is shown in Fig. 5A and D; note the surrounding astroglial (Fig. 5B and E) and microglial (Fig. 5C and F) responses indicating that the hemorrhage likely occurred several days prior to necropsy. There was also focal neuronal necrosis in the parenchyma immediately underlying the hemorrhages. In a second fetus, a small hemorrhage was seen...
in the cerebrum. In a third fetus, there were minor hemorrhages in both locations, accompanied by thickened meninges containing IBA-1-IR macrophages.

Quantitative Morphometric Analysis in the Cerebrum

Overall the density of GFAP-IR cells was not different (P > 0.05) between control and EtOH fetuses in the gray matter (21.2 ± 19.8/mm² vs. 12.1 ± 9.5/mm²), subcortical white matter (216.4 ± 51.8/mm² vs. 213.8 ± 31.9/mm²), or deep white matter (208.4 ± 33.6/mm² vs. 182.2 ± 31.0/mm², Fig. 6A). The density of ramified IBA-1-IR cells in deep white matter was also similar (202.9 ± 44.7/mm² vs. 196.7 ± 43.7/mm²) in both groups (Fig. 6B). The mean area of microglial infiltration (amoeboid morphology) in the white matter of the cerebrum was not different between EtOH and control groups (P = 0.413, Fig. 6C).

The optical density of MBP-IR showed no significant difference between control and EtOH groups in subcortical white matter (0.11 ± 0.02 vs. 0.11 ± 0.03) or in deep white matter (0.09 ± 0.02 vs. 0.09 ± 0.03, Fig. 6D). The density of TUNEL-positive cells was not different between groups in the gray matter (0.56 ± 0.73/mm² vs. 0.19 ± 26/mm²) or white matter (2.10 ± 2.20/mm² vs. 1.62 ± 1.24/mm², Fig. 6E). The percentage of parenchyma occupied by blood vessels was not different in gray matter (5.55 ± 0.98% vs. 5.90 ± 0.72%, P = 0.44) or deep white matter (3.86 ± 0.98% vs. 3.33 ± 0.80%, Fig. 6F).

Quantitative morphometric analysis in the cerebellum. The optical density of GFAP-IR in cerebellar white matter was not different between EtOH and control groups (0.278 ± 0.004 vs. 0.284 ± 0.006).

Proinflammatory Cytokine Gene Expression

In the fetal white matter there were no differences in mRNA gene expression levels of proinflammatory cytokines IL-1β (1.00 ± 0.08 vs. 0.98 ± 0.15), IL-6 (1.00 ± 0.31 vs. 1.19 ± 0.18), IL-8 (1.00 ± 0.20 vs. 0.86 ± 0.29), and TNF-α (1.00 ± 0.29 vs. 0.85 ± 0.07), as shown in Fig. 7. Similar observations were made in the placenta (Fig. 7) apart from a significant increase in TNF-α expression in EtOH exposed placentas.

DISCUSSION

In this study, we demonstrate that repeated daily exposure to EtOH in late gestation induces mild, transient changes in blood chemistry in both the ewe and fetus, but with no changes in baseline values. In the mother, arterial lactate concentration increased at 1–8 h, arterial glucose concentration decreased at 1–4 h, and arterial pH decreased at 2–4 h. In the fetus, SaO₂ decreased at 6–10 h, and PaO₂ decreased at 10 h; arterial lactate increased at 4–10 h. There was little evidence of significant fetal brain injury or fetal growth restriction, apart from a nonsignificant trend for a lower ponderal index suggestive of thinness. The lack of effect on fetal growth or brain development is surprising in view of our previous findings of significant effects on both of these following 3 days of similar EtOH exposure (10, 14). We recognize that other regimens of EtOH exposure, involving levels higher than those used in the present study can cause neuronal loss, particularly of cerebellar Purkinje cells (41).

Plasma EtOH Concentrations

As in our previous studies (10, 14), PEC in ewes and fetuses reached maximal values 1 h after the start of infusions and were undetectable by 10 h. The maximal maternal and fetal PEC were also similar to those of our previous studies (10, 14) and were equivalent to maximal levels reached in women weighing 60–70 kg, 1 h after drinking 3–4 USA standard drinks over 1 h; a USA standard drink contains 0.5 fluid ounces or 11.7 g EtOH (25). As there was no significant difference between PEC in the ewe and fetus at any time, our study confirms that EtOH distributes unimpeded between the maternal and fetal circulations (4, 36). The earlier suggestion that EtOH can accumulate in the amniotic fluid, prolonging the duration of fetal exposure relative to the mother (3), is clearly incorrect.

Fetal Blood Gases

In the present study, a mild delayed reduction in fetal blood oxygenation was shown by a decrease in SaO₂ at 6–10 h and PaO₂ at 10 h following EtOH infusion. As these changes were not accompanied by evidence of changes in maternal oxygenation, the observed transient reduction in fetal blood oxygenation was likely a result of a mild decrease in transplacental oxygen delivery. However, unlike studies in which higher levels of EtOH were infused into ewes, we found no evidence of fetal hypercapnia and acidemia (9). We were unable to assess placental function in this study, but we did observe that the expression of proinflammatory cytokines IL-1β, IL-6, and IL-8 in the placenta was not affected by prior EtOH exposure. TNF-α mRNA gene expression was increased in EtOH fetuses suggestive of a persistent inflammatory response in the placenta.
In the past, fetal hypoxemia was proposed as a cause of EtOH-induced alterations in development observed in children with FASD (1), although subsequent studies (30, 33, 35) have not supported a role of fetal hypoxemia. The present study confirms that consuming 3.8 USA standard drinks can cause a mild decrease in fetal arterial oxygenation, but only after a prolonged period and at a time when EtOH is virtually absent. The decrease in fetal blood oxygenation may therefore be a result of an action by a metabolite of EtOH, such as acetaldehyde. Acetaldehyde concentrations in fetal blood and amniotic fluid can peak at about 50% of maternal blood acetaldehyde concentrations following EtOH exposure (19). Whatever the cause of the delayed reduction in fetal arterial oxygenation, it is unlikely to be sufficient to contribute to EtOH-induced altered development or injury.

We observed a mild but significant reduction in both maternal and fetal PaCO2, which began from the infusion period (0–1 h) and continued until 4–6 h after the start of infusion. As this mild hypocapnia occurred in both groups it is likely a result of events related to the time of day, which could have induced maternal hyperventilation. The infusion period coincided with daily feeding of the sheep; the infusion protocol itself necessitated the close proximity of staff to the ewes prior to, during, and following the infusion period. A decrease in PaCO2 of this magnitude would be unlikely to affect fetal development.

**Fetal Blood Glucose Concentrations**

The observed decrease in maternal blood glucose concentration could be a result of inhibition of gluconeogenesis by EtOH (22, 39). Gluconeogenesis and glycolysis rely on NAD⁺ availability and may be impaired following EtOH exposure as NAD⁺ is used for oxidative metabolism of both EtOH and acetaldehyde. In sheep and humans, fetal glucose is almost entirely acquired from the mother via the placenta (2, 18) and one would expect that decreases in maternal blood glucose would be reflected by a similar decrease in fetal blood glucose. The lack of substantial hypoxemia in the fetus following EtOH exposure could be a result of decreased fetal glucose utilization; EtOH is known to inhibit...
fetal glucose transporters GLUT1 and GLUT3 (15, 34). Decreased fetal glucose uptake and utilization could explain the observed trend toward a reduction in ponderal index in our EtOH-treated fetuses.

**Fetal Blood Lactate Concentration**

Following EtOH infusions, arterial lactate concentration increased in both ewes (by 300% at 4 h) and fetuses (by 70%...
at 8–10 h). In human adults, EtOH metabolism usually leads to an increase in blood lactate concentrations and lactate acidosis (23). The EtOH-induced increase in blood lactate concentration in adult humans is likely due to changes in lactate production and elimination brought about by EtOH and is probably mediated by decreased NAD$^{+}$ bioavailability. In pregnancy, EtOH metabolism in the fetal-maternal unit occurs primarily in the maternal liver (4, 36), and therefore the greater percentage increase in blood lactate concentration observed in our ewes compared with their fetuses (300% vs. 70%) probably reflects the primary role that the ewe plays in EtOH metabolism in the maternal-fetal unit.

We observed that maternal blood lactate concentration peaked at 4 h after infusion onset, whereas fetal lactate concentration peaked later (8 h). The more rapid and proportionally greater increase in maternal lactate levels supports the concept that most of the lactate synthesis occurs in the maternal liver, due to its major role in EtOH metabolism. It is thought that lactate can distribute readily across the placenta, primarily by noncarrier-mediated passive diffusion (17). Thus it is likely that the synthesis of lactate in the ewe and its diffusion across the placenta to the fetus is responsible for the more gradual increase in blood lactate concentration in the fetus.

Fetal Cardiovascular Function

In the present study, there were no significant changes in fetal MAP or HR following daily EtOH exposure, nor was baseline MAP or HR affected. Previous studies have shown alterations in placental perfusion following EtOH infusion (12, 33), but they involved higher EtOH concentrations than in our study. EtOH infusions can affect maternal and fetal cardiovascular function in a dose-dependent manner (9). In sheep, EtOH exposure has been shown to increase fetal and maternal HR and maternal MAP, while mildly decreasing fetal MAP by ~5 mmHg, but only with a very high peak blood EtOH concentration (0.26 g/dl) (9). These studies suggest that the effects of EtOH exposure on cardiovascular function are complex and likely depend on the peak level of blood EtOH concentration attained. In the present study, we achieved peak PEC of ~0.11–0.12 g/dl in the ewe and fetus, which may not have been sufficient to affect MAP or HR.

Fetal Growth

High levels of EtOH exposure in gestation have been associated with fetal growth restriction and delayed weight gain in childhood (16, 24). Similarly, in pregnant sheep exposed to EtOH both prior to and throughout gestation, near-term fetuses were lighter, shorter, and had decreased brain weight compared with controls (31). Our recent study of 3 days of EtOH exposure showed evidence of restricted fetal growth (3). In contrast, in our present study, fetal body weights were unaffected by daily EtOH exposure over 39 days; this suggests that the fetus is capable of adapting or recovering from the inhibitory effects of EtOH on growth over 39 days. However, we did observe that the relative fetal heart weight tended to be increased after EtOH exposure. We also observed a trend for the ponderal index to be reduced in EtOH-exposed fetuses, suggesting that daily EtOH exposure over 39 days may cause fetuses to be thinner in relation to body length. In rats, EtOH exposure can decrease the overall percentage of fetal body fat by ~23% (7). Such a decrease in body fat could account for the trend for a reduction in ponderal index; however, the fat content of our fetuses was not examined.

Fetal Brain Development

The regimen of EtOH exposure that we used, apart from minor hemorrhages and associated cortical injury in three fetuses, did not result in any gross morphological effects on the fetal cerebrum or cerebellum in terms of brain weight or overt injury. Neither was there any difference in the extent of forebrain myelination and vascularization, evidence of cell death, or distribution of microglia (ramified or amoeboid) and astrocytes within the gray matter and white matter. We recognize that there could be alterations at the microstructural level in relation to synaptic development or receptor distribution and efficacy but these analyses were beyond the scope of the present study.

We cannot rule out the possibility that neuronal loss may have occurred as reported recently in a nonhuman primate model. A 35% reduction in the number of neurons in the frontal cortex was observed in fetal vervet monkeys exposed to EtOH (equivalent to 3–5 standard drinks), four times a week during the third trimester equivalent (5). Furthermore, it has been shown that a single binge-like exposure in the third trimester in the macaque triggered widespread apoptosis in the fetal brain within 1 h of the conclusion of alcohol administration (13). Stereological techniques as used in other studies (5) or alternatively, examining the immediate effects of alcohol exposure (13) would be required to detect such alterations, but these approaches were beyond the scope of our study. It also appears that during prenatal and postnatal life, primates (human and nonhuman) are more sensitive to the pharmacological and toxicological effects of EtOH compared with other mammalian species, including the rat, mouse, guinea pig, and sheep, in view of the higher EtOH concentration required to produce a particular functional or structural effect in these species (8, 36).

In relation to subarachnoid hemorrhages, prenatal alcohol exposure has been shown to affect endothelium-dependent vascular function in some arterial beds in fetal sheep (29) and to increase stiffness of the aorta in children (26). Thus it is possible that alcohol-induced alterations to cerebral vessels during development could affect their structural integrity, increasing the possibility of hemorrhages. Bleeding could be associated with the potential release of iron which could catalyze free radical-mediated injury in underlying brain tissue.

We observed no changes in mRNA expression levels of the inflammatory cytokines IL-1β, IL-6, IL-8, and TNF-α in cerebral white matter at 24 h following the last EtOH infusion. This finding is in accordance with our observation that microgliosis was not evident in cerebral white matter, and demonstrates that the fetal immune response is not affected in this model of daily EtOH exposure, at least not when assessed 24 h after the final infusion.

In our previous study, in which EtOH was administered daily for 3 days at 116 DGA (0.8 of gestation), we observed evidence of brain injury in 50% of fetuses, including an increase in microglial infiltration into white matter (10). Therefore it surprised us that the fetal brain in the present study appeared to be relatively unaffected by a much longer period of
daily EtOH exposure; our findings suggest that there was some degree of recovery from the initial insult or that tolerance to EtOH may have occurred. The apparent adaptation to, or recovery from, the neurotoxic effects of EtOH in the fetus during late gestation may be due, in part, to the plasticity of the developing ovine brain during the ontogenetic period of the brain growth spurt (11). It is also possible that repeated EtOH exposure leads to the development of cellular-molecular mechanisms of resistance to the neurotoxic actions of EtOH.

Prenatal EtOH exposure can cause postnatal behavioral abnormalities in the absence of neurological damage (38). Unpublished studies in our laboratory using the same fetal EtOH exposure protocol, but allowing lambs to be born and raised to 9 wk postnatal age, showed no observable behavioral differences between groups, although specific testing involving, for example visual recognition (20) or spatial orientation tasks (6), were not applied. Developmental milestones including the amount of time taken to stand, walk, and suckle were not different between groups (K. Kenna, unpublished observations).

Conclusions

We conclude that daily EtOH exposure during the third trimester equivalent of ovine gestation, sufficient to raise maternal and fetal PEC to a maximum of ~0.11 g/dL, induces mild, transient, physiologic changes in the mother and fetus with no observable overt damage to the fetal brain with the exception of minor subarachnoid hemorrhages in three of eight cases. As we were unable to assess neuronal numbers and/or axonal and dendritic growth, the absence of subtle alterations in fetal brain development cannot be entirely discounted.

Perspectives and Significance

The relatively mild changes in fetal physiological variables and absence of overt effects in the fetal brain suggest that the fetus is capable of adapting to an environment of repeated EtOH exposure during late gestation. However, the presence of small subarachnoid hemorrhages in some alcohol-exposed fetuses is of concern.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES