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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Running title: Effects of daily ethanol exposure on the fetus

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Abstract

High levels of ethanol (EtOH) consumption during pregnancy adversely affect fetal development; however the effects of lower levels of exposure are less clear. Our objectives were to assess the effects of daily EtOH exposure (3.8 USA standard drinks) on fetal-maternal physiological variables and the fetal brain, particularly white matter. Pregnant ewes received daily intravenous infusions of EtOH (0.75g/kg maternal body-weight over 1h, 8 fetuses) or saline (8 fetuses) from 95-133 days of gestational age (DGA, term ~145 DGA). Maternal and fetal arterial blood was sampled at 131-133 DGA. At necropsy (134 DGA) fetal brains were collected for analysis. Maternal and fetal plasma EtOH concentration reached similar maximal concentration (~0.11 g/dL) and declined at the same rate. EtOH infusions produced mild reductions in fetal arterial oxygenation but there were no changes in maternal oxygenation, maternal and fetal PaCO₂, or in fetal mean arterial pressure or heart rate. Following EtOH infusions, plasma lactate levels were elevated in ewes and fetuses, but arterial pH fell only in ewes. Fetal body and brain weights were similar between groups but relative heart weight was increased after EtOH exposure. In 3/8 EtOH exposed fetuses there were small subarachnoid hemorrhages in the cerebrum and cerebellum associated with focal cortical neuronal death and gliosis Overall, there was no evidence of cystic lesions, inflammation, increased apoptosis or white matter injury. We conclude that daily EtOH exposure during the third trimester-equivalent of ovine pregnancy has modest physiological effects on the fetus and no gross effects on fetal white matter development.

Abstract: 250 words

Keywords: brain injury, blood gas status, oxygenation, lactate, arterial pressure
Introduction

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 one-hour 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 white matter development (32). Each daily EtOH infusion raised maternal and fetal plasma EtOH concentrations to a maximal value of 0.11 g/dL, the equivalent of a 60-70 kg woman consuming 3-4 USA standard drinks in one hour (25). Five days after the initial EtOH exposure there was evidence
of injury and altered development of white matter in the sub-cortical 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 three days led to a reduction in fetal body weight of 19% compared with controls, and reductions in maternal plasma insulin like growth factor (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 three days would have more severe effects on fetal growth and brain development, or whether fetal adaption to this more chronic exposure would occur.

In the present study, the period of daily EtOH exposure during ovine pregnancy was increased from three 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 which 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 95-133 days of gestation (DGA), which 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.

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 i.v. infusions of either EtOH or saline, and sampling maternal arterial blood. At 126 DGA, the ewes underwent further aseptic surgery for implantation of catheters into a fetal brachial artery catheter 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 catheterised. Following each period of surgery, ewes were administered i.v. antibiotics (Ampicillin, 500mg, Douglas Pharmaceuticals, Australia) for 3 days.

Experimental protocol: The ewes were randomly assigned to either an EtOH infusion group (EtOH, n=8 fetuses) or a control group (Controls, n=8 fetuses). The EtOH group received daily, one-hour infusions of EtOH (i.v.), starting at approximately 09.00h each day, from 95 – 124 DGA and from 128 – 133 DGA; infusions were not performed on 125-7 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 two weeks so that the EtOH dose could be adjusted for maternal weight gain. This dose was chosen to obtain maximal plasma EtOH concentrations (PEC) of 0.11 - 0.12 g/dL, and was based on previous studies (10, 38). Control ewes received an equivalent volume of saline at
the same time each day. During the infusion periods, ewes were allowed free access to water and food. Control ewes were provided with extra daily feed (~120 gram) to compensate for the additional calories contained in EtOH (7.1 calories/gram). Ewes were fed at approximately 08.30h each day.

Over three days (131-133 DGA), maternal (3mL) and fetal (3mL) arterial blood samples were taken immediately before each infusion (time = 0 h) and at 1, 2, 4, 6, 8, 10, and 24h after the infusion onset; a total of 24mL of blood was removed from the fetus on each of the 3 days. Red blood cells were reinfused into the fetus if hemoglobin concentration (Hb) dropped below 30%, however this was only required in one fetus from the EtOH group. PEC was analysed using the Dade Behring Dimension RxL Clinical Chemistry System (10, 14). Fetal and maternal blood samples were also analysed (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, NSW, Australia) from 131 to 133 DGA. Fetal HR was derived from the arterial pressure waveform. The digitised data were subsequently analysed over 10 min intervals to obtain hourly values. Arterial pressure was adjusted for amniotic pressure.

Ewes and fetuses were euthanised 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 euthanized with sodium pentobarbital (130 mg/kg i.v.) administered i.v. to the ewe. Fetuses were weighed and crown-to-rump length (CRL), thoracic girth, forelimb length and head length measured; the fetal ponderal index (body weight ÷ CRL³) was calculated. Major organs
were collected and weighed. The fetal brain was removed and placed in 4% paraformaldehyde in 0.1M phosphate buffer (pH7.4).

*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 (5mm thick) was also taken from each cerebellar vermis. All blocks were embedded in paraffin wax. Six to eight sections (10μm) 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 (H&E) 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 anti-glial 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] 1:1500, 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 anti-myelin basic protein (MBP; 1:200; Chemicon International) was used. All sections were incubated in the appropriate secondary antibodies (1:200) for 90 minutes 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 minutes in a microwave oven.
Sections from each lobe were stained with DeadEnd Colorimetric TUNEL System (Promega, 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 v6.2; Media Cybernetics, Frederick, MD). In each slide, a field of view refers to an area of 0.2mm². 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: 1) cells immunoreactive for GFAP were counted in four randomly selected fields in the gray matter (GM), two fields in subcortical white matter (SWM) and two fields in deep white matter (DWM). A mean value (8 fields for GM, 4 for SWM and 4 for DWM) was calculated for each animal. 2) IBA-1-IR cells with a ramified (resting) morphology were counted in five randomly selected fields in DWM in the parietal and temporal lobes and ten fields in the occipital lobe; a mean value (n=15 fields) was calculated for each animal. The area of activated IBA-1-IR cells with an amoeboid (activated) morphology was measured in 1 parietal and 2 occipital sections for each animal and a mean value calculated. 3) To determine the intensity of immunoreactivity for MBP in SWM and DWM, optical density was measured using Image Pro Plus software. Four fields were chosen randomly from each lobe and a mean value was calculated for each animal. 4) Cells positive for TUNEL staining were counted in five randomly selected fields and the area of GM and WM measured in each field in each of the lobes. A mean value for TUNEL positive cells in GM and WM was calculated. 5) The proportion of cerebral parenchyma occupied by blood vessels
was estimated by point counting (40). An increase in density above control levels suggests that cerebral hypoxemia has occurred. Three randomly selected fields in the GM and DWM of each lobe were analysed, and a mean value for each of 6 fetuses calculated.

Quantitative morphometric analysis in the fetal cerebellum. The optical density of GFAP-IR in the WM was determined as described above for the cerebrum.

Gene expression analysis: Relative mRNA levels of the pro-inflammatory cytokines interleukin (IL)-1β, -6, -8 and tumor necrosis factor (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 analysed. Total RNA was extracted using an RNeasy kit (Qiagen, Australia), treated with DNase (Qiagen, Australia) and reverse-transcribed into cDNA (M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant Kit; Promega, USA). 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, USA), 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 (that 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 analyzed using the ΔCT (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 131-133 DGA); data were not different on each of these days. The mean data from each fetus were then analysed from the start of the infusion period (time = 0) to 10 h post-infusion 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 analyse morphometric, necropsy, neuropathology and gene expression data. Statistical significance was accepted at p<0.05. Data are presented as mean ± SEM.

Results

Plasma ethanol 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 non-measurable levels by 8 hours after the end of the EtOH infusion (Figure 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 (Figure 2A) and SaO₂ (data not shown) were not different between EtOH and control groups. Similarly, maternal PaCO₂ following EtOH infusions was not different from values in controls (Figure 2B). In both EtOH and control groups maternal PaCO₂ decreased over time (Figure 2B), with minimal values at 4-6h (P_{time} = 0.001). With respect to maternal arterial pH (Figure 2C),
although the overall treatment effect was not significant ($P_{\text{treat}} = 0.236$), there was a significant interaction between treatment and time ($P_{\text{treat\times time}} = 0.001$). At 2h after the start of the infusions, arterial pH was significantly lower in EtOH ewes than in controls (7.435±0.013 vs 7.487±0.007) and remained significantly lower at 4h.

After EtOH infusions, maternal blood glucose concentration fell below control values, but there was only a non-significant trend for an overall treatment effect ($P_{\text{treat}} = 0.089$). However, there was a highly significant interaction between treatment and time ($P_{\text{treat\times time}} = 0.000$, Figure 3A); glucose concentrations in EtOH ewes were significantly lower than in the control ewes by 44%, 46% and 33% at 1, 2 and 4h, respectively, after the start of the EtOH infusion. Maternal blood lactate concentration increased significantly at the end of the EtOH infusion (1h) and remained elevated at 2, 4 and 6h compared to the control group (Figure 3B). The maximal maternal blood lactate concentration was 1.56±0.19 mmol/L, and occurred at 4 h after the start of the EtOH infusion.

**Fetal physiological data:** The changes in fetal PaO2 and SaO2 after the EtOH infusions were significantly different in the EtOH exposed and control groups (Figure 4A, 4B). Fetal PaO2 slowly fell after the EtOH infusion and at 10h reached 20.7±0.5 mmHg which was significantly (17%) lower than in controls (Figure 4A). Fetal PaO2 had returned to control values at 24h after the EtOH infusion. Fetal SaO2 also fell after the EtOH infusion and was, respectively, 16% and 21% lower than in the control group at 8 and 10h (Figure 4B); the minimal SaO2 (55.2±2.4%) occurred at 10h, as with fetal PaO2. There was no difference between the EtOH treated and control groups in fetal PaCO2 or arterial pH (Figure 4C, 4D); 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 24h.

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$_{treat*time} = 0.001$, 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 and ewes. In EtOH-exposed fetuses, lactate concentrations were significantly higher than in control fetuses at 4, 6, 8 and 10h after the start of the infusions (Figure 3D). In EtOH fetuses, the maximal lactate concentration of 2.0±0.2, measured at both 8h and 10h, was not significantly higher than the maximal value in ewes (1.6±0.2 mmol/L at 4h).

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 BPM.
Necropsy data: At necropsy (134-135 DGA) the body weight of EtOH fetuses was 3.8±0.2 kg (n=8) which was not different from that of control fetuses (4.1±0.2 kg, n=8, Table 2). There 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±0.7 vs. 7.2±0.5, 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 WM 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 cerebellum and cerebrum. An example is shown in Figure 5A; note the surrounding astroglial (Fig 5B, E) and microglial (Fig 5C, 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 the 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 EtOH and control fetuses in the gray matter (21.2±19.8 /mm² vs. 12.1±9.5 /mm²), subcortical WM (216.4±51.8 /mm² vs. 213.8±31.9 /mm²) or deep WM (208.4±33.6 /mm² vs 182.2±31.0 /mm², Figure 6A). The density of ramified IBA-1-IR cells
in deep WM was also similar (202.9±44.7 /mm² vs 196.7±43.7 /mm²) in both groups (Figure 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, Figure 6C).

The optical density of MBP-IR showed no significant difference between EtOH and control groups in subcortical WM (0.11±0.02 vs. 0.11±0.03) or in deep WM (0.09±0.02 vs. 0.088±0.03, Figure 6D). The density of TUNEL positive cells was not different between groups in the GM (0.2±0.3 /mm² vs. 0.6±0.7 /mm²) or WM (2.1±2.2 /mm² vs. 1.6±1.2 /mm², Figure 6E). The percentage of parenchyma occupied by blood vessels was not different in GM (5.55±0.98 % vs. 5.90±0.72 %, p=0.44) or deep WM (3.86±0.98 % vs. 3.33±0.80 %, Figure 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 Figure 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 1-8h, arterial glucose concentration decreased at 1-4h, and arterial pH decreased at 2-4h. In the fetus, SaO₂ decreased at 6-10h and PaO₂ decreased at 10h; arterial lactate increased at 4-10h. At necropsy the relative heart weight was 22% greater after EtOH exposure; this is unlikely to be related to arterial pressure as MAP was not different between groups. There was little evidence of significant fetal brain injury or fetal growth restriction, apart from a non-significant 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 recognise 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 (PEC):** As in our previous studies (10, 14), PEC in ewes and fetuses reached maximal values one hour 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, one hour after drinking 3-4 USA standard drinks over one hour; a USA standard drink contains 0.5 fluid ounces or 11.7 g EtOH (25). As there was no significant difference between plasma EtOH concentrations 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 hours and PaO₂ at 10 hours 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 trans-placental 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 pro-inflammatory 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 U.S.A standard drinks can cause a mild decrease in fetal arterial oxygenation, but only after a prolonged period, 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 (0-1h) and continued until 4-6h 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 utilisation could explain the observed trend towards 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 hours) and fetuses (by 70% at 8-10 hours). 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 to 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 4h after infusion onset whereas fetal lactate concentration peaked later (8h). 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 non-carrier-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 about 5mmHg, but only with a very high peak blood EtOH concentration (0.26g/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 about 0.11-0.12g/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 human 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 was 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 vascularisation, evidence of cell death or distribution of microglia (ramified or amoeboid) and astrocytes within the gray 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 non-human 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 one hour 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 non-human) are more sensitive to the pharmacological and toxicological effects of ethanol compared to other mammalian species, including the rat, mouse, guinea pig and sheep, in view of the higher ethanol 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 catalyse free-radical mediated injury in underlying brain tissue.

We observed no changes in mRNA expression levels of the inflammatory cytokines IL-1β, IL6, IL8 and TNFα in cerebral white matter at 24 hours 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 hours after the 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 ethanol in the fetus during late gestation may be due, in part, to the plasticity of the developing ovine brain during the ontogenic period of the brain growth spurt (11). It is also possible that repeated ethanol exposure leads to the development of cellular-molecular mechanisms of resistance to the neurotoxic actions of ethanol.

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 weeks 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 (unpublished findings).
**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 approximately 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 3/8 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.
Acknowledgments

The authors are grateful for the assistance of, Natasha Blasch, Steven Gray and Alex Satragno.

Grants

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**Figure Legends**

**Figure 1:** Maternal (filled circles, n=8) and fetal (open squares, n=7) plasma EtOH concentrations (PEC) measured following a one hour maternal EtOH infusion (0.75 g EtOH/kg maternal body wt; shading). For each animal, plasma EtOH concentration (PEC) was measured on days 131-133 of gestation and values averaged. Data are presented as mean ± SEM. * p <0.05, compared to t=0h for both maternal and fetal data.

**Figure 2:** Changes in (A) PaO₂ (mmHg), (B) PaCO₂ (mmHg) and (C) pH in ewes that received a one hour maternal EtOH infusion (filled circles, n=8) or a one hour saline infusion (controls, open squares, n=7). Shaded bar indicates EtOH or saline infusion. Data were obtained on days 131-133 of gestation and values averaged. Data are presented as mean ± SEM. * p <0.05, compared to t=0h for both treatment groups. † p<0.05, EtOH compared to saline.

**Figure 3:** Changes in (A) maternal glucose (mmol/L) (B) maternal lactate (mmol/L), (C) fetal glucose (mmol/L) and (D) fetal lactate (mmol/L) following a one hour maternal EtOH infusion (filled circles, n=5-7) or one hour saline infusion (open squares, n=5-7). Data were obtained on days 131-133 of gestation and averaged. Data are presented as mean ± SEM. † p<0.05, EtOH compared to saline.

**Figure 4:** Changes in (A) PaO₂ (mmHg), (B) SaO₂ (%) , (C) PaCO₂ (mmHg) and (D) pH in fetuses that were exposed to a one hour maternal EtOH infusion (filled circles, n=7) or one
hour saline infusion (open squares, n=5). Data were obtained on days 131-133 of gestation and averaged. Data are presented as mean ± SEM. † p<0.05, EtOH compared to saline.

**Figure 5:** Subarachnoid hemorrhages in the cerebrum (A – C) and cerebellar vermis (D – F) following EtOH exposure in the same fetus. H&E-stained section of the parietal cortex (A) shows hemorrhage and associated astroglial (GFAP-IR; B) and microglial (IBA-1-IR; C) responses. The box in (A) shows the region represented in B and C. H&E-stained section of the cerebellum (D) shows parenchymal hemorrhage with destruction of the cortex and associated astroglial (GFAP; E) and microglial (IBA-1; F) responses. Hemorrhages in (A) and D are signified by “H”. The arrows in (B) and (E) indicate astrogliosis and those in (C) and (E) indicate microgliosis. Scale bars: (A) 200 μm, (B - F) 100 μm.

**Figure 6:** Quantitative morphometric analysis of the forebrain for control (open columns, n=8) and EtOH (closed columns, n=8) fetuses. (A) Density of GFAP IR cells in grey matter (GM), sub-cortical white matter (SWM), deep white matter (DWM). (B) Density of IBA-1 IR cells in DWM. (C) Microglial infiltration in WM. (D) Optical density of MBP IR product in SWM and DWM. (E) Density of TUNEL positive (apoptotic) cells in GW and WM. (F) Percent blood vessels occupying tissue in GM and DWM. Data are presented as mean ± SEM.

**Figure 7:** Qualitative real time PCR mRNA gene expression analysis of cytokines in the cerebral white matter of control (open columns, n=8) and EtOH-exposed (closed columns, n=8) fetuses. mRNA gene expression analysis of (A) IL-1β, (B) IL-6, (C) IL-8 and (D) TNF-α. Data are presented as mean ± SEM.
References


Maternal

A

1. Glucose (mmol/L)

P_{treat} = 0.089
P_{time} = 0.000
P_{treat\times time} = 0.000

B

2. Lactate (mmol/L)

P_{treat} < 0.001
P_{time} < 0.001
P_{treat\times time} < 0.001

Fetal

C

1. Glucose (mmol/L)

P_{treat} = 0.340
P_{time} = 0.001
P_{treat\times time} = 0.001

D

2. Lactate (mmol/L)

P_{treat} = 0.025
P_{time} = 0.001
P_{treat\times time} = 0.004

Time (hours)
Table 1: The nucleotide sequences for each forward and reverse primer (5’-3’) 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.

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<th>Gene</th>
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<th>Forward</th>
<th>Reverse</th>
<th>Temp.</th>
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<td>CAG GGT TCT CGC TCT TGC</td>
<td>ACT GGC GGC ACA TAT TGA G</td>
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<tr>
<td>IL-1β</td>
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<td>CGA TGA GCT TCT GTG TGA TG</td>
<td>CTG TGA GAG GAG GTG GAG AG</td>
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<tr>
<td>IL-6</td>
<td>NM_001009392</td>
<td>CGC AAA GGT TAT CAT CAT CC</td>
<td>CCC AGG AAC TAC CAC AAT CA</td>
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<tr>
<td>IL-8</td>
<td>NM_001009401</td>
<td>CCT CAG TAA AGA TGC CAA TGA</td>
<td>TGA CAA CCC TAC ACC AGA CC</td>
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<tr>
<td>TNF-α</td>
<td>NM_001024860</td>
<td>AGT CTG GGC AGG TCT ACT TTG</td>
<td>GGT AAC TGA GGT GGG AGA GG</td>
<td>60°C</td>
<td>4 ng/μg</td>
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Table 2: Necropsy data for ethanol (n=7) and saline (n=9) treated fetuses. EtOH was infused via the ewe for one hour (0.75 g EtOH/kg maternal body wt) from 95–124 DGA and from 127–133 DGA. * indicates p<0.05 between groups, CRL = crown-to-rump length, TG = thoracic girth, FLL = forelimb length, HL = Head length, PI = ponderal index (body weight ÷ CRL³), BW = body weight. Data shows mean ± SEM.

<table>
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<tr>
<th></th>
<th>Ethanol (5 Males, 3 Females)</th>
<th>Control (5 Males, 3 Females)</th>
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<tr>
<td>Gestational Age</td>
<td>133.8 ± 0.2</td>
<td>133.9 ± 0.1</td>
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<tr>
<td>Body Weight (kg)</td>
<td>3.8 ± 0.3</td>
<td>4.1 ± 0.2</td>
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<tr>
<td>CRL (cm)</td>
<td>48.3 ± 1.4</td>
<td>47.6 ± 1.3</td>
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<tr>
<td>TG (cm)</td>
<td>33.5 ± 1.3</td>
<td>35.1 ± 1.1</td>
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<tr>
<td>FLL (cm)</td>
<td>42.5 ± 0.9</td>
<td>41.5 ± 1.3</td>
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<tr>
<td>Head Length (cm)</td>
<td>12.8 ± 0.3</td>
<td>13.0 ± 0.3</td>
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<tr>
<td>PI (g/cm³ x 100)</td>
<td>3.4 ± 0.2</td>
<td>3.8 ± 0.3</td>
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<tr>
<td>Heart (g)</td>
<td>32.1 ± 2.5</td>
<td>28.9 ± 1.5</td>
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<td>Heart/BW (g/kg)</td>
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<td>7.2 ± 0.5</td>
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<td>Lungs (g)</td>
<td>142.1 ± 17.8</td>
<td>133.3 ± 6.6</td>
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<tr>
<td>Lungs/BW (g/kg)</td>
<td>35.4 ± 2.9</td>
<td>32.8 ± 1.6</td>
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<tr>
<td>Liver (g)</td>
<td>108.2 ± 15.2</td>
<td>104.6 ± 9.4</td>
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<tr>
<td>Liver/BW (g/kg)</td>
<td>27.7 ± 3.1</td>
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<td>Kidneys (g)</td>
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<td>11.5 ± 1.2</td>
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<td>Kidneys/BW (g/kg)</td>
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<td>Adrenal/BW (g/kg)</td>
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<td>0.056 ± 0.006</td>
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<tr>
<td>Brain (g)</td>
<td>48.3 ± 2.1</td>
<td>50.9 ± 1.9</td>
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<tr>
<td>Brain/BW (g/kg)</td>
<td>13.5 ± 0.9</td>
<td>12.5 ± 0.5</td>
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