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# Effects of Early Daily Alcohol Exposure on Placental Function and Fetal Growth in a Rhesus Macaque Model

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## Abstract

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**Background—**Prenatal alcohol exposure is the most common cause of birth defects and intellectual disabilities and can also increase the risk of stillbirth and negatively impact fetal growth.

**Objective—**The purpose of this study is to determine the effect of early prenatal alcohol exposure on non-human primate placental function and fetal growth. We hypothesize that early chronic prenatal alcohol will alter placental perfusion and oxygen availability that adversely effects fetal growth.

**Study Design—**Rhesus macaques self-administer 1.5g/kg/day ethanol (n=12) or isocaloric maltose-dextrin (n=12) daily preconception through the first 60 days of gestation (G60, term is G168). All animals were serially imaged with Doppler ultrasound to measure fetal biometry, uterine artery volume blood flow and placental volume blood flow. Following Doppler ultrasound, all animals also underwent both BOLD-MRI to characterize placental blood oxygenation and DCE-MRI to quantify maternal placental perfusion. Animals were delivered by cesarean section for placental collection and fetal necropsy at G85 (n=8), G110 (n=8), or G135 (n=8). Histologic and RNA-Seq analysis was performed on collected placental tissue.

**Results—**Placental volume blood flow was decreased at all gestational time points in ethanol-exposed vs. control, but most significantly at G110 by Doppler ultrasound ($p<0.05$). A significant decrease occurred in ethanol-exposed vs. control animals in total volumetric blood flow on DCE-MRI at both G110 and G135 ($p<0.05$) as well as a global reduction in $T_2^*$, high blood deoxyhemoglobin concentration, throughout gestation ($p<0.05$). Similarly, evidence of placental ischemic injury was notable by histologic analysis, which revealed a significant increase in microscopic infarctions in ethanol-exposed, not control, largely present at mid to late gestation. Fetal biometry and weight were decreased in ethanol-exposed vs. control, but not significant. Analysis with RNA-Seq suggests involvement of the inflammatory and extracellular matrix response pathways.

**Conclusions—**Early chronic prenatal alcohol exposure significantly diminishes placental perfusion at mid- and late-gestation and significantly decreases the oxygen supply to the fetal vasculature throughout pregnancy, associated with microscopic placental infarctions in the non-human primate. Although placental adaptations may compensate for early environmental perturbations to fetal growth, placental blood flow and oxygenation is reduced, consistent with evidence of placental ischemic injury.

## CONDENSATION:

First trimester alcohol exposure alters placental function and fetal growth in a rhesus macaque model

### Keywords

Fetal alcohol spectrum disorder; fetal alcohol syndrome; maternal drinking; placental perfusion and oxygenation; prenatal alcohol exposure

## INTRODUCTION

Alcohol freely crosses the placenta and can accumulate in the fetus at levels comparable to maternal blood alcohol concentrations.[1] Prenatal alcohol exposure increases the risk of preterm birth, stillbirth, decreased fetal growth and fetal alcohol spectrum disorder (FASD), the most common non-genetic cause of cognitive impairment in the United States.[2,3] Currently, there are no approved drugs to treat FASD or established tools to prevent adverse outcomes. Among pregnant women in the United States, approximately 10% have consumed any alcohol the past 30 days,[4] resulting in greater than three quarters of a million alcohol-exposed fetuses.[5-7]

The placenta occupies a central role in supporting normal fetal growth and development during pregnancy.[8] Prior studies have suggested that placental dysfunction may contribute to intrauterine growth restriction (IUGR) in FASD,[2] but the mechanisms and specific vasoactive effects of alcohol linking placental dysfunction to disrupted fetal growth remains an area of ongoing scientific exploration. Antenatal ethanol exposure has been previously shown to induce apoptosis in human placental trophoblast cells, disrupt trophoblast cell motility, and potentially affect uterine spiral artery remodeling.[9-13] Prior pregnant animal studies using ovine[14] and baboon[15-18] models have demonstrated abnormal uterine and cerebral blood flow following acute ethanol exposure and in a rat model impaired uterine artery vasodilation from chronic binge drinking.[19]

Non-human primate (NHP) fetal ontogeny, placental structure, and ethanol absorption and metabolism more closely resembles that of humans than other animal models.[17] We recently developed a NHP model of first trimester ethanol consumption that generated pregnancies from control animals and dams that drank 1.5g/kg of ethanol daily (~6 standard drinks for humans).[21] Fetal brain maturation was characterized with *in utero* magnetic resonance imaging (MRI) and *ex vivo* slice electrophysiology, which revealed that cerebellar and brainstem fetal growth is diminished, in this model of FASD[22] Moreover, fetal diffusion MRI indicated altered maturation of motor-related white matter fiber systems, and these findings were corroborated with altered synaptic development in cortical and striatal regions found by electrophysiology[22]. Previously, a pilot investigation that demonstrated feasibility and greater sensitivity of *in utero* MRI directed at the placenta to detect the effects of early-pregnancy drinking on placental function and fetal development[23] over standard clinical doppler-ultrasound (Doppler-US). Placental blood flow was measured using dynamic contrast-enhanced MRI (DCE-MRI)[24] and oxygen exchange was quantified through analysis of water $T_2^*$ values via the blood oxygen level-dependent (BOLD) effect.[25]

Utilizing these *in vivo* MRI methods at gestational day 110 (G110, term is ~168 days) and G135, we observed that early gestation alcohol exposure decreased both placental perfusion and fetal oxygen supply mid-gestation and was associated with both decreased fetal and brain weight.[23] However, the underlying mechanisms contributing to the observed altered placental function and fetal development were not explored in this prior work. The primary objective of this study was to utilize the complete set of animals in which placental tissue is available, including at an additional gestational timepoint (G85), to evaluate the adverse effects of early, chronic prenatal alcohol exposure on placental outcomes and fetal growth.

Our second objective was to study the effects of prenatal ethanol exposure on placental histology and gene expression to identify mechanisms underlying placental dysfunction detectable using non-invasive imaging approaches.

## MATERIALS AND METHODS

### Experimental Design

All protocols were approved by the Institutional Animal Care and Use Committee of the Oregon National Primate Research Center, and guidelines for humane animal care were followed. The generation of pregnancies in the NHP FASD model has been previously published.[21] This study focuses on the subset of time-mated pregnant rhesus macaques (n=24) consisting of 12 control and 12 ethanol-exposed animals that underwent placental collection at time of delivery. Dams were trained to orally self-administer daily either 1.5g/kg/day of 4% ethanol solution (~6 drinks/day) or an isocaloric control fluid with training being initiated at least 4 months prior to undergoing time-mated breeding with plasma estradiol sampling[26-28] (Figure 1). The day of peak plasma estradiol was defined as gestational day 0 (G0). Each pregnant animal continued daily drinking of 1.5 g/kg/day ethanol ad lib until G60, at which point access to ethanol, or isocaloric solution, was removed (Figure 1). All animals underwent Doppler-US followed by MRI consisting of $T_2$ and DCE measurements and after imaging, immediate cesarean section delivery with placenta collection and fetal necropsy at G85 (n=8), G110 (n=8) or G135 (n=8) (Figure 1). Collected placental tissue was processed in liquid nitrogen for molecular analysis, RNAlater for RNA-Seq, and formalin fixation for histology.

### Imaging

**Doppler-US—**All ultrasounds were performed by a single sonographer (J.O.L) using image-directed pulsed and color Doppler equipment (GE Voluson 730) with a 5- to 9-MHz sector probe. Animals were sedated by intramuscular administration of 10 mg/kg ketamine (Henry Schein Animal Health®) and maintained on a portable anesthesia delivery system providing $O_2$ with 1.5% isoflurane. Doppler waveform measurements for the uterine artery (Uta) and umbilical artery were performed using machine-specific software. The following measurements were obtained: pulsatility index (PI), velocity time integral (VTI), and fetal heart rate (HR) to calculate uterine artery blood flow ($cQ_{Uta}$) and placental volume blood flow ($cQ_{UV}$) as previously described.[23,29-33] $cQ_{Uta}$ was calculated and corrected by maternal weight as: $cQ_{Uta}$= VTI x CSA (Uta cross-sectional area) x HR. [29-33] Placental volume blood flow ($cQ_{UV}$) was calculated as: mean velocity ($V_{mean}$) x CSA x 60. [29-33]

**Placental MRI—**Immediately following ultrasound, MRI studies were performed on a 3T Siemens TIM-Trio scanner (Erlangen, Germany) as previously published.[23] Following localization of the placenta and acquisition of anatomic images in the coronal and axial planes, axial 2D multislice spoiled multiecho gradient echo images (TR=418 ms, flip angle=30°, 256x72 matrix, 96 slices, 1.5 mm isotropic spatial resolution) spanning the entire uterus were acquired at six in-phase echo times (TE=4.92, 9.84, 19.68, 29.52, 36.90, and 44.28 ms) for $T2^*$ measurements, and $T_1$ was measured with the variable flip angle (VFA) method.[34] After acquisition of VFA data, 150 3D SPGR images were acquired for DCE-

MRI (TR=2.00 ms, TE=0.72 ms, flip angle=20°, acquisition time of 3.64 seconds), with field of view and resolution matched to the VFA images. Ten baseline images were acquired prior to intravenous injection of 0.1 mmol/kg of gadoteridol contrast reagent (Prohance®, Bracco Diagnostics Inc, Princeton, NJ) at a rate of 30 mL/min using a syringe pump (Harvard Apparatus, Holliston, MA). Anatomic and multiecho imaging was performed during expiratory breath holding, while DCE-MRI data were acquired during ventilated breathing. Physiological monitoring of pulse rate, arterial blood oxygen saturation, and end-tidal $CO_2$ partial pressure was performed throughout, with no deviations from normal ranges observed in these parameters. BOLD and DCE-MRI analyses were performed as previously described.[23,34,35]

**Placental histology—**Formalin fixed paraffin-embedded histologic sections were stained with hematoxylin and eosin and reviewed by a single placental pathologist (T.K.M.) blinded to exposure and outcomes. Tissue sections were scored for any signs of infection and classic histologic features of maternal vascular mal-perfusion, including infarctions and/or accelerated villous maturation.[36]

**Statistical Analysis—**Data are expressed as mean ± SD. All animals (n=24) were analyzed and differences between ethanol-exposed and controls at G85, G110 and G135 were tested by a 2-way ANOVA with post-hoc Tukey comparison, significance was designated at $p<0.05$. The $T_2^*$ results were evaluated using a two-sample Kolmogorov-Smirnov test.

## Gene Expression

**RNA Isolation and Quality Assessment—**Dissected placenta tissue samples (n=24) in RNAlater (ThermoFisher Scientific) were delivered to the OHSU Gene Profiling Shared Resource, where phenol-chloroform extraction was performed followed by RNA isolation using the RNeasy Mini kit (QIAGEN). RNA integrity and size distribution were assessed using a 2100 Bioanalyzer (Agilent Technologies). Four samples were excluded due to suboptimal RNA yield.

**RNA Sequencing—**RNA-seq libraries were prepared using the TruSeq with Ribosomal Depletion kit (Illumina). The amplified product was profiled on the TapeStation (Agilent). Libraries were quantified using real time PCR (Kapa Biosystems) and run on a HiSeq 2500 (Illumina). The resulting base call files were converted to fastq files using bcl2fastq (Illumina)

**Gene-level Differential Expression Analysis—**Differential expression analysis utilized standard operating procedures established by the ONPRC Bioinformatics & Biostatistics Core. The quality of the raw sequencing files was evaluated using FastQC[37] combined with MultiQC (http://multiqc.info/).[38] Trimmomatic[39] was used to remove any remaining Illumina adapters. Reads were aligned to Ensembl's mmul8 along with its corresponding annotation, release 87. The program STAR[40] (v2.7.3a) was used to align the reads to the genome and RNA-SeQC[41] was utilized to ensure alignments were of sufficient quality.

The differential expression analysis was performed in the open-source software R.[42] Gene-level raw counts were filtered to remove genes with extremely low counts in many samples following the published guidelines,[43] normalized using the trimmed mean of M-values method (TMM),[44] and transformed to log-counts per million with associated observational precision weights using voom[45] method. Gene-wise linear models with primary variables treatment group, gestational stage and their interaction, adjusting for sex and technical factors (RNA processing and sequencing batch)were employed for differential expression analyses using limma with empirical Bayes moderation[46] and false discovery rate (FDR) adjustment.[47]

**RNA Pathway Analysis—**A total of 12,879 genes were considered expressed in the 18 placental samples dataset after filtering. To adjust for multiple comparisons, an FDR adjusted p-value of < 0.2 was used to test for significance because of the small sample size and degree of biological variation. As is common in longitudinal NHP studies with relatively high variability and small group sizes, few genes were identified as differentially expressed at FDR < 0.2 in the comparisons between ethanol and control or comparisons between gestational time points, except in ethanol-exposed samples at G135 vs G85. To overcome this limitation, pathway-based patterns were explored to look for effects on gene networks, pathways, and biological processes due to maternal ethanol consumption. Pathway analysis[48] was performed in Ingenuity Pathway Analysis (IPA). (QIAGEN Inc., https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). The core analysis option in IPA was used to perform pathway analysis using an unadjusted p-value < 0.05 and fold change > 1.5 for all of the pairwise post-hoc comparisons.

## RESULTS

### Growth parameters

Ultrasound measurements of fetal biometry were not significantly different between fetuses exposed to ethanol and controls at all gestational ages (Table 1). There was no significant difference in fetal birth weight at time of delivery in ethanol-exposed fetuses versus control animals at G85 (p=0.5), G110 (p=0.07) and G135 (p=0.1) (Table 2). Maternal weights and fetal gender ratios were not significantly different across treatment groups (Table 2). All major fetal organs were examined by a veterinary pathologist and no gross structural anomalies were noted in ethanol-exposed pregnancies.

### Placental perfusion and oxygenation

Both cQuta and cQuv was smaller in the ethanol-exposed group compared to controls at G110 (p<0.05), but differences were not seen at G85 or G135 (Table 3). Uterine artery pulsatility indices were increased in ethanol-exposed animals, but was not statistically significant at all gestational time points (Table 3). Similarly, there was a trend of increased umbilical artery pulsatility indices suggestive of increased placental vascular impedance across all time points that was significant at G85 (p<0.05) (Table 3).

Maternal perfusion of the placental intervillous space was evaluated using MRI and multiple irregularities were seen in ethanol-exposed animals compared to controls. Using DCE-MRI,

comprehensive maternal perfusion through spiral arteries is quantified as total volumetric blood flow. This was found to be significantly lower ($p<0.05$) at G110 and G135 in ethanol-exposed group versus controls (Table 3), consistent with the semi-quantitative measures of cQuta at G110.

Placental oxygen supply was assessed through analysis of water $T_2$ values. In control placentas at all three timepoints, MR image voxels proximal to spiral artery sources of oxygenated maternal blood are characterized by relatively long $T_2$ (Figure 2), as previously described.[35] At greater distances from the spiral arteries, the concentration of deoxyhemoglobin is higher, secondary to fetal oxygen uptake, resulting in decreased $T_2$*. The histograms shown in Figure 2 summarize placental $T_2$*, demonstrating a statistically significant reduction in $T_2$* across gestation, but most prominently at G85 ($p=0.01$) compared with G110 ($p=0.04$) and G135 ($p=0.03$) in the ethanol-exposed cases compared to controls.

## Placental histology

Placental pathology demonstrated increased frequency of microscopic (<1.0cm) infarctions in placentas exposed to ethanol (5/12, $p<0.05$) compared with controls (0/12) (Figure 3). These infarctions were predominantly seen at G110 (2/4) and G135 (2/4) (Figure 3). Larger placental infarctions were also seen in a few cases, but only in ethanol-exposed placentas (Figure 3). There was no histologic evidence of infection, increase in placental villi maturation or findings of chorangiosis. Placental weights were not different amongst different treatment groups at all three pregnancy timepoints (Table 2).

## RNA Sequencing

Pairwise comparisons between sample groups were performed, and the comparison with the most significantly differentially expressed genes was G135 vs. G85 in the ethanol-exposed samples: 505 upregulated genes were identified in G135 compared to 397 upregulated genes in G85 (FDR<0.2) (Figure 4). For the pathway analysis of G135 vs. G85 in ethanol-exposed placentas, there were 1356 analysis-ready molecules with p-value < 0.05 and fold-change > 1.5. IPA identified 71 significant pathways. Of note, Ethanol Degradation IV, Oxidative Ethanol Degradation III, and Ethanol Degradation II pathways have negative z-scores indicating predicted inhibition of these pathways at G135 vs. G85 in ethanol-exposed samples but not controls.

Comparing ethanol-exposed samples to control samples at each timepoint, pathway analysis was used to look for patterns affecting multiple genes of related function, utilizing thresholds of unadjusted p-value < 0.05 and fold change > 1.5. Pathways with $p < 0.001$ are shown in Figure 5. Many of these contain genes involved with extracellular matrix remodeling and inflammation.

# COMMENT

## Principal Findings

Ethanol exposure through the first 60 days post-conception, akin to the first trimester in human pregnancies, reduced both placental blood flow and oxygenation, with more dramatic effects on placental perfusion at G110 and G135 (Table 3). These findings were accompanied by increased placental pathology and associated with differences in the expression of genes related to inflammation and maintenance of the extracellular matrix. Additionally, ethanol-exposed placentas demonstrated inhibition of gene pathways related to ethanol degradation at G135 compared to G85, not seen in control placentas.

## Clinical Implications

Ethanol readily crosses the placenta and accumulates in the fetus at concentrations proportionate to maternal blood levels within an hour.[1] Consistent with our earlier study[21] and existing literature,[49] we have shown in a larger cohort that early, chronic prenatal alcohol exposure plays an important role in altered placental perfusion and function. These findings suggest that placental impairment secondary to early prenatal ethanol exposure is a possible contributor to the previously reported altered fetal brain development[22] and decreased cerebral artery blood flow[16-18] since the placenta provides oxygen and transit of essential metabolites for routine fetal nervous system development.[50]

Our analysis by RNA-Seq suggests involvement of inflammatory and extracellular matrix response pathways contributing to the observed decreased placental perfusion and fetal oxygen availability. These findings played a role in the significant presence of placental microinfarctions, suggestive of reduced functional villi, in ethanol-exposed animals only. Early ethanol exposure may have altered these pathways and contributed to endothelial dysfunction in the placental vasculature. Similarly, in a NHP model of Western Style diet (WSD), we had found that consumption of a WSD during pregnancy exacerbated placental inflammation, reduced uterine blood flow and significantly increased the frequency of both placental infarctions and stillbirth.[29] Additionally, RNA-Seq findings of downregulated Ethanol Degradation II, III, IV genes at G135 compared to G85 in ethanol-exposed placentas, but not controls suggests ethanol metabolism pathway inhibition. This is possibly another adaptive placental response to early-ethanol exposure to protect against ethanol toxicity. Other studies have also similarly reported altered ethanol degradation pathways in gestational alcohol exposure.[51]

Decreased placental perfusion and fetal oxygen availability occurred most significantly at mid- to late-gestation, but was lowest at G110, 50 days after ethanol cessation. Although in a smaller subset of animals, early alcohol exposure appeared to impact fetal birth weight[23], in this larger cohort, preserved fetal growth and placental weight was observed, suggestive of the NHP placenta's adaptive capabilities to early transient environmental perturbations[19,52] when compared with chronic prenatal insults throughout gestation.[25,53,54] In spite of this compensatory response, the reduced placental oxygenation at G135 combined with the presence of placental microinfarctions suggest persistent placental injury that could further disrupt fetal development in ongoing pregnancies.

## Research Implications

The placenta has significant functional reserve to maintain adequate fetal nutrient delivery during pregnancy. However, when a critical threshold for placental compensation is reached,[23,53] it can result in complications such as fetal growth restriction with increased fetal and neonatal mortality and morbidity.[19] Thus, it is important to determine a non-invasive method to identify pregnancies at risk of hemodynamic alterations before the fetus is affected.

RNA-Seq was also performed to examine gene expression differences suggestive of pathways important to placental development and function that are affected by early chronic ethanol exposure. Our findings suggestive of inflammatory and extracellular matrix remodeling pathway involvement reveal mechanistic targets for future investigation for early intervention in pregnancies affected by first trimester alcohol exposure prior to pregnancy detection.

## Strengths and Limitations

This study builds upon our prior earlier work[23] by using a larger cohort to investigate of the biological effects of early prenatal alcohol exposure on placental function and fetal growth. Also, by adding a gestational time point and utilizing placental histology and gene expression analysis, we were able to establish correspondences between *in vivo* imaging findings and identified placental pathology and RNA-Seq results.

This animal model provides precise control over experimental variables such as gestational age and quantity and timing of ethanol exposure throughout gestation. Uncertainties in such variables confound studies of human subjects. All placentas were collected at time of cesarean section delivery prior to onset of spontaneous labor from pregnancies with similar environmental exposures, including diet. Thus, the direct effects of early alcohol consumption were more readily assessed compared to human studies with weaker correlative measures.

Limitations of this study were the animal cohort size and cross-sectional study design, chosen to avoid confounds associated with repeated isoflurane exposure, used for sedation during MRI procedures. Future studies from our group will focus on longitudinal assessments within the same animal to allow for more direct interrogation of this placental acclimatization phenomenon. Additionally, with RNA Seq analysis our sample size did not provide the power to detect differentially expressed genes with small effect sizes between the experimental groups at a single timepoint.

## Conclusions

Early chronic prenatal ethanol exposure significantly impairs maternal placental perfusion and oxygen supply to the fetal vasculature throughout pregnancy resulting in associated placental pathology in voluntary-drinking rhesus macaques. These alterations are detectable early in gestation using newly developed non-invasive imaging techniques, demonstrating increased sensitivity over standard Doppler-US. Abnormal gene expression patterns related to inflammation and the extracellular matrix are associated with the observed functional

deficits. Placental adaptations were able to compensate for the adverse effect to placental blood flow and oxygenation by maintaining normal fetal growth through G135 in this experimental cohort. At this time, there is no treatment for FASD or the adverse fetal affects from prenatal alcohol exposure. Thus, it is important for women thinking of conceiving or those who are pregnant to adhere to the recommendations by ACOG to abstain from alcohol consumption.

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## AJOG AT A GLANCE

**Why was the study conducted?**

**a.** This study was conducted to determine the effects of chronic maternal first trimester binge drinking on placental function and fetal growth.

**What are the key findings?**

**a.** Early chronic prenatal alcohol exposure significantly diminishes placental perfusion at mid- and late-gestation and significantly decreases the oxygen supply to the fetal vasculature throughout pregnancy in the rhesus macaque.

**What does this study add to what is already known?**

**a.** This study established correspondences between reduced comprehensive placental blood flow and oxygen availability on *in-vivo* MRI, increased placental microinfarctions on histology and upregulated inflammatory and extracellular matrix remodeling pathways by RNA-Seq in pregnancies with early alcohol exposure.

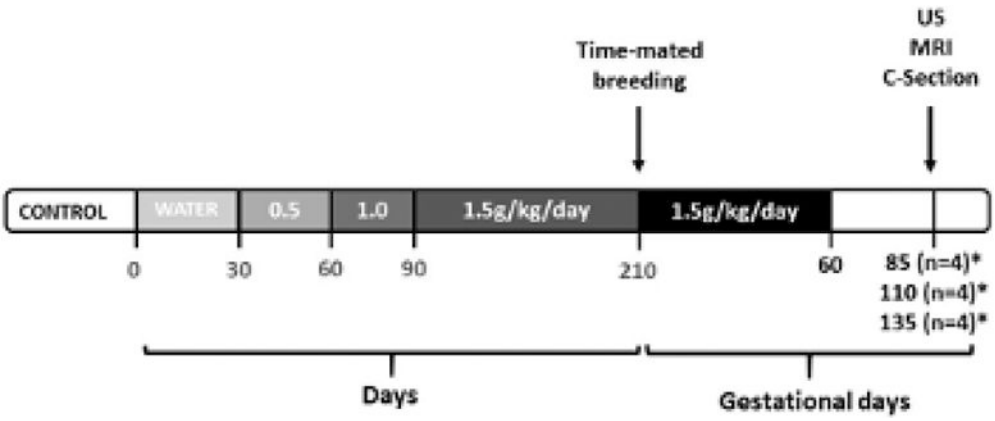


**Figure 1. Study Design Overview.**

Timeline of the experimental design indicating that the ethanol-exposed animals were trained to self-administer ethanol with incremental dosing increase until reaching a dose of 1.5g/kg/day (~6 drinks/day) which was then maintained through the first 60 days post-conception before being discontinued. The age-matched control group (*) self-administered an isocaloric control fluid also through gestational day 60 (G60, term is ~168 days). Ultrasound and MRI were performed at G85, G110 and G135 with immediate cesarean delivery following imaging at G135.

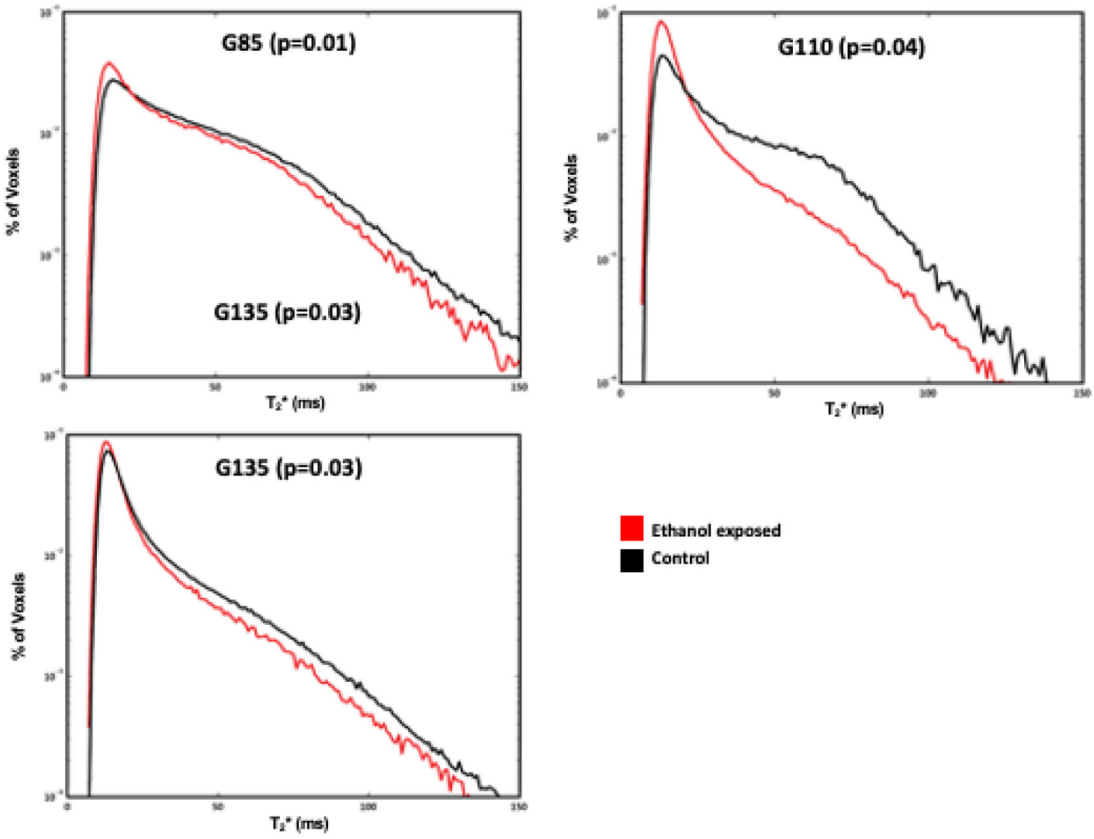


**Figure 2. Histogram plot of $T_2$* versus percent of placental voxels displayed for ethanol-exposed (red) vs. control animals (black) at G85, G110 and G135.**

Ethanol-exposed animals had a smaller fraction of large $T_2$* values compared to controls across all time points, demonstrating decreased fetal oxygen availability in the former.


**Figure 3. Placental histologic changes associated with early maternal ethanol exposure.**

(A) Representative control H&E stained placental section at G110 compared with microscopic infarctions (asterix) seen in (B) ethanol-exposed animals. Magnification is 50x.

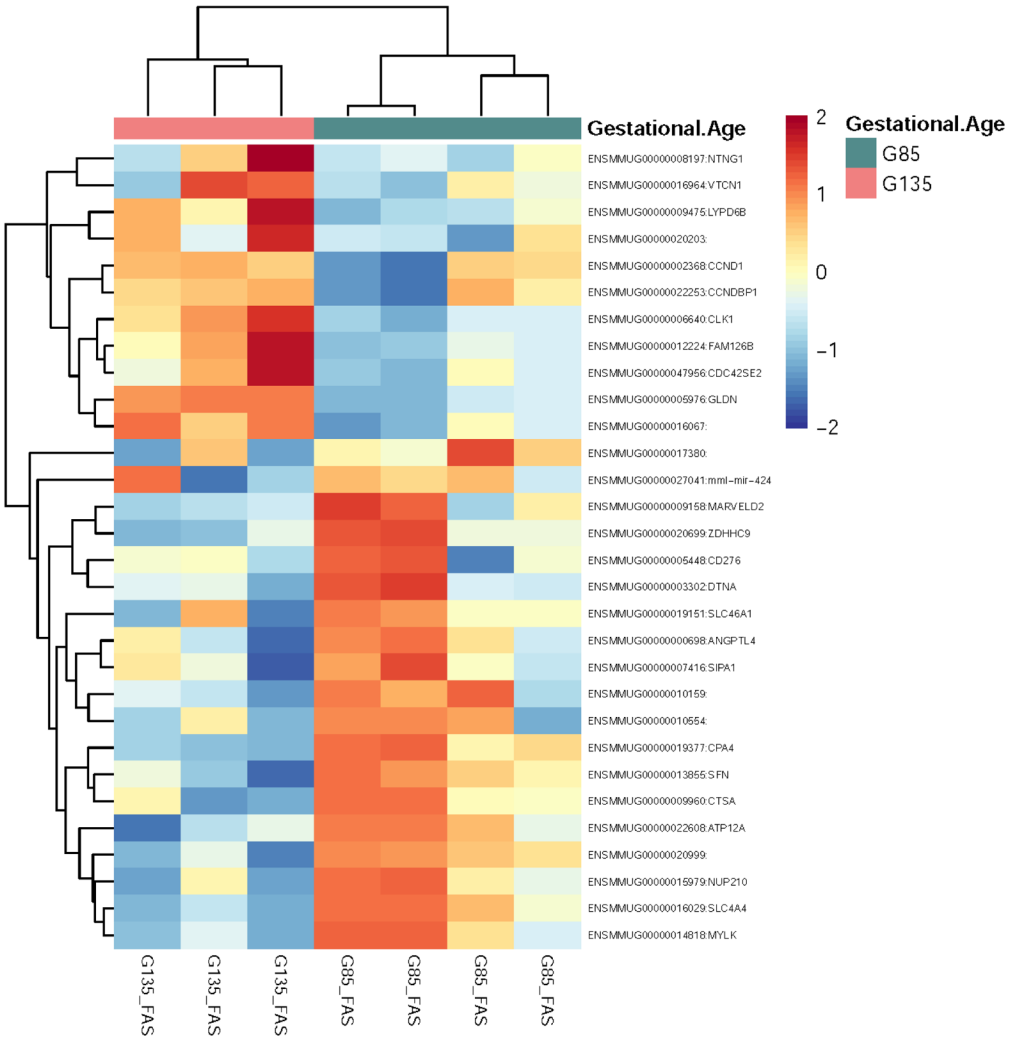


**Figure 4. RNA Seq Heatmap.**

Heatmap of top 50 differentially expressed genes by FDR adjusted p-value between FAS G135 and G85.

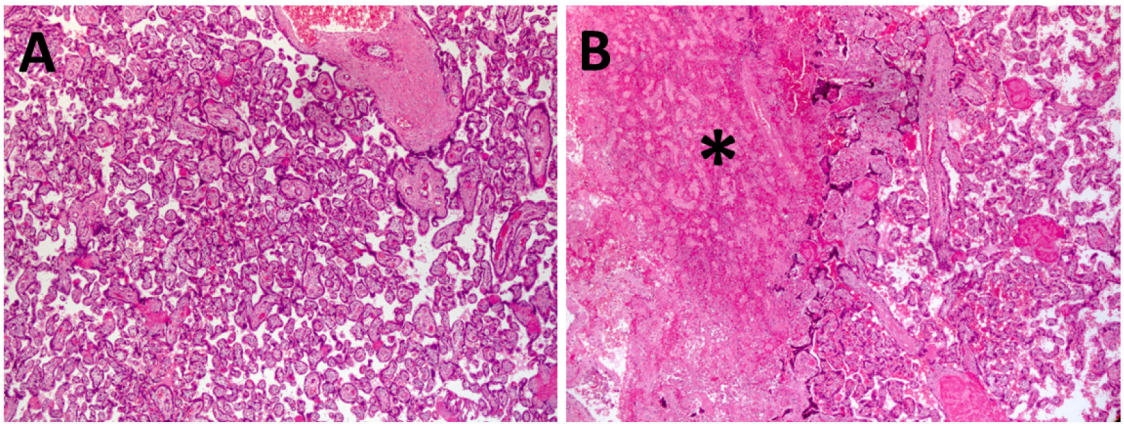


**Figure 5. RNA Seq Pathway Analysis.**

Significant pathways ($p<0.001$) when comparing ethanol-exposed (FAS) and control (CON) samples at each timepoint and between timepoints within each treatment group.

**Table 1.**

Fetal biometry

| Parameter | Gestational day 85 | | Gestational day 110 | | Gestational day 135 | |
|---|---|---|---|---|---|---|
| | Control (n=4) | Ethanol (n=4) | Control (n=4) | Ethanol (n=4) | Control (n=4) | Ethanol (n=4) |
| BPD (mm) | 29.4 ± 1.4 | 28.3 ± 1.6 | 38 ± 1.7 | 36.7 ± 3.5 | 44.5 ± 0.8 | 42.6 ± 2.6 |
| AC (mm) | 9 ± 0.3 | 8.9 ± 0.5 | 12 ± 0.4 | 11.6 ± 0.7 | 13.7 ± 0.4 | 12.9 ± 1.4 |
| FL (mm) | 17.9 ± 1.2 | 6.8 ± 0.9 | 28.5 ± 0.9 | 27.3 ± 1.2 | 36.6 ± 1.9 | 36 ± 0.4 |

Definition of abbreviations:

BPD = biparietal diameter

AC = abdominal circumference

FL = femur length

2-Way ANOVA used with post-hoc Tukey comparison test. Data are means ± SD.

* $p<0.05$

**Table 2.**

Maternal, fetal birth, and placental weights

| Parameter | Gestational day 85 | | Gestational day 110 | | Gestational day 135 | |
|---|---|---|---|---|---|---|
| | Control (n=4) | Ethanol (n=4) | Control (n=4) | Ethanol (n=4) | Control (n=4) | Ethanol (n=4) |
| Maternal weight (kg) | 7.1±0.8 | 6.8±0.9 | 8.1±1.9 | 7.8±1.5 | 7.8±1 | 8.3±0.9 |
| Fetal weight (g) | 81.7±7.8 | 77.8±10.3 | 209±23.8 | 183±16.7 | 334±9.5 | 323±17 |
| Placental weight (g) | 49.4±15 | 42.9±3.6 | 77±14.7 | 64.6±4.1 | 81±6.2 | 82.4±8.6 |
| Fetal sex (male:female) | 3:1 | 2:2 | 1:3 | 1:3 | 1:3 | 1:3 |

2-Way ANOVA used with post-hoc Tukey comparison test. Data are means ± SD.

* $p<0.05$

**Table 3.**

Doppler ultrasound and Dynamic contrast-enhanced MRI measurements of placental function and oxygenation

| Parameter | Gestational day 85 | | Gestational day 110 | | Gestational day 135 | |
|---|---|---|---|---|---|---|
| | Control (n=4) | Ethanol (n=4) | Control (n=4) | Ethanol (n=4) | Control (n=4) | Ethanol (n=4) |
| Uterine artery PI | 0.7±0.1 | 0.9±0.2 | 0.6±0.1 | 0.8±0.1 | 0.8±0.3 | 0.85±0.1 |
| Umbilical artery PI | 1.7±0.3 | 2.4±0.3* | 1.3±0.2 | 1.6±0.3 | 1.1±0.06 | 1.4±0.3 |
| cQuta (ml/min/kg) | 22.3±7.3 | 14±6.3 | 41±23 | 22±11.5* | 26.5±14 | 26±1.4 |
| cQuv | 17.7±2.2 | 9.8±3.6 | 24.6±4.8 | 15.1±0.7* | 31.5±7.1 | 28.9±13.7 |
| Total Placental blood flow (ml/min)§ | 289±120 | 121±27 | 681±39.6 | 285±142* | 559±190 | 414±221* |

Definition of abbreviations:

§ = obtained by DCE-MRI

PI = pulsatility index, VTI = velocity time integral

CSA (cross section of uterine artery) = $\pi$(diameter/2)$^2$

Vmean (mean velocity) = 0.5 x maximum umbilical vein velocity

cQuta (uterine artery blood flow) = VTI x CSA x HR adjusted for maternal weight

cQuv (placental volume blood flow) = Vmean x CSA x 60

2-Way ANOVA used with post-hoc Tukey comparison test. Data are means ± SD.

* $p<0.05$