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Distribution of Phosphatidylethanol in Maternal and Fetal Compartments after Chronic Gestational Binge Alcohol Exposure

Vishal Naik, M.S, Raine Lunde-Young, M.S, Josue Ramirez, B.S, Jehoon Lee, PhD, Jayanth Ramadoss, PhD*

Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, 77843, USA.

Abstract

Background: Phosphatidylethanol (PEth) is a promising biomarker for gestational alcohol exposure. Studies show PEth accumulation in maternal and fetal blood following alcohol exposure, however, distribution of specific PEth homologues (16:0/18:1, 16:0/18:2, 16:0/20:4) in maternal and fetal blood is unknown. Additionally, PEth levels in highly vulnerable FASD targets in maternal and fetal compartments remain unexplored. We hypothesized that all three major PEth homologues will be detectable in the maternal and fetal blood, the maternal uterine artery (a reproductive tissue that delivers oxygen and nutrients to feto-placental unit), and fetal brain regions following gestational binge alcohol exposure, and that homologue distribution profiles will be tissue-specific.

Methods: Pregnant rats received once-daily orogastric gavage of alcohol (Alcohol; BAC 216 mg/dl@4.5g/kg/day; BAC 289 mg/dl@6g/kg/day) or iso-caloric maltose-dextrin (Pair-fed control) from gestation day (GD) 5–20 or 21. Following chronic exposure, maternal and fetal tissues were analyzed for PEth homologue concentrations utilizing LC-MS/MS technology.

Results: All three PEth homologues were detected in alcohol-exposed maternal blood, fetal blood, maternal uterine artery, and fetal brain regions (hippocampus, cerebral cortex, cerebellum). In both maternal and fetal blood, respectively, PEth 16:0/18:2 was more abundant compared to 16:0/18:1 ($P<0.0001$, ~66%↑; $P=0.0159$, 20.4%↑) and 16:0/20:4 ($P=0.0072$, ~25%↑; $P=0.0187$, 19.4%↑). Maternal PEth 16:0/20:4 was ~42% higher than 16:0/18:1 ($P=0.0015$). Maternal PEth 16:0/18:2 and 16:0/20:4 were ~25%↑ and ~20%↑ higher than in fetal blood ($P<0.05$). No homologue differences were detected in the maternal uterine artery. In all fetal brain regions, PEth 16:0/18:1 was significantly higher ($P<0.0001$) than 16:0/18:2 (~48–78%↑) and 16:0/20:4 (~31–62%↑) concentrations. PEth 16:0/20:4 was ~18% higher than 16:0/18:1 ($P<0.05$) in the fetal hippocampus and cortex.

Conclusion: All major PEth homologues were detected in maternal and fetal blood following chronic gestational binge alcohol exposure; homologue distribution profiles were tissue specific.

*Corresponding Author: Jayanth Ramadoss, PhD, 336 VID1, Hwy 60, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station TX 77843-4466, USA. Phone: (979) 458-3280; Fax (979) 845-6544; jramadoss@cvm.tamu.edu.

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This study also provides insights into PEth accumulation in critical FASD targets, specifically the maternal uterine artery and fetal brain.

Keywords

PEth; biomarker; pregnancy; maternal; fetal

Introduction

Fetal Alcohol Spectrum Disorder (FASD) is an umbrella term describing the range of structural, functional, and neurodevelopmental abnormalities associated with developmental alcohol exposure (Bertrand et al., 2005; CDC, 2018; Sokol, 2003). Prenatal alcohol exposure continues to be a leading cause of preventable developmental disabilities and birth defects in the United States (“NIH,” 2018). A recent conservative estimate placed 2–5% of US school-going children within this spectrum (May et al., 2018, 2009, “NIH,” 2018). Early identification of alcohol exposure during pregnancy would warrant timely intervention and would facilitate development of preemptive therapeutic strategies for at-risk individuals and their developmental outcomes. However, a major challenge opposing early identification of gestational alcohol exposure is the current lack of reliable bio-markers that possess both a high specificity for alcohol and a long detection window (Bakhireva and Savage, 2011; Bearer, 2001; Luginbühl et al., 2019).

Alcohol metabolites that persist in tissues indicate prior alcohol exposure and may also act as mediators of alcohol-induced toxicity during fetal development (Frezza et al., 1990; Swift, 2003). Various direct and indirect biomarkers (i.e. ethyl glucuronide, ethyl sulfate, phosphatidylethanol, fatty acid ethyl esters, etc.) have been proposed to assess gestational alcohol exposure, however, each have individual advantages and limitations (i.e. in their sensitivity, specificity, and post-exposure detection window) (Bakhireva and Savage, 2011). Phosphatidylethanol (PEth), a direct, non-oxidative alcohol metabolite, is formed in the body specifically during alcohol metabolism via transphosphatidylation of phosphatidylcholine (PC) by the enzyme phospholipase D (PLD) (Alling et al., 1984). PEth has been widely described as an ideal candidate biomarker for detecting alcohol exposure, as it exhibits marked specificity for alcohol (~100%) and its stability allows it to be detected in blood even after 29 days of abstinence in heavy drinkers (Helander and Zheng, 2009; Jastrzbska et al., 2016; Javors et al., 2016). There are about 48 known homologues of PEth, each identified based on the number of carbons and double bonds in their fatty acid chain (Figure 1). Each PEth homologue mirrors their parent PC homologue from which they form. Of all the PEth homologues detected in blood following alcohol exposure, 16:0/18:1 and 16:0/18:2 are the most abundant (38% and 24%, respectively), closely followed by 16:0/20:4 (13%). Each of these three PEth homologues has a distinct pharmacokinetic profile signature (Gnann et al., 2010; Helander and Zheng, 2009; Lopez-Cruzan et al., 2018). Owing to their abundance and the uniqueness of their pharmacokinetic profiles, characterizing their distribution profile in maternal and fetal blood may reveal insights into the type of alcohol exposure pattern (Javors et al., 2016; Lopez-Cruzan et al., 2018). Additionally, identifying the PEth homologue distribution profiles in different maternal and fetal tissues may show direct tissue-specific effects of alcohol metabolism.

Although few studies have examined PEth formation in maternal and fetal blood following gestational alcohol exposure (Bakhireva et al., 2014; Baldwin et al., 2015; Kwak et al., 2014), the current study is the first to examine and compare the distribution of the three major PEth homologues 16:0/18:1, 16:0/18:2, and 16:0/20:4 in both maternal and fetal blood and brain regions with well-known vulnerability to alcohol, following a chronic gestational binge exposure paradigm. Gestational alcohol exposure has been implicated in disrupting pregnancy-induced adaptations and function of the maternal uterine artery, a vessel which supplies all nutrients and oxygen essential for healthy fetal development (Gundogan et al., 2008; Naik et al., 2018; Ramadoss and Magness, 2012; Subramanian et al., 2014). Therefore, we selected the uterine artery as a tissue of interest in the maternal compartment. Additionally, the current study is the first to quantify and compare PEth homologue distribution in the fetal brain regions, which are widely studied FASD targets, following gestational alcohol exposure. We hypothesize that all three major PEth homologues 16:0/18:1, 16:0/18:2, and 16:0/20:4 will be detectable in the maternal blood, fetal blood, maternal uterine artery, and fetal brain regions following gestational binge alcohol exposure, and that the homologue distribution profiles will be tissue-specific.

Materials and Methods

All experimental procedures were in accordance with National Institutes of Health guidelines (NIH Publication No. 85–23, revised 1996) with approval by the Animal Care and Use Committee at the Texas A&M University. Timed pregnant Sprague–Dawley rats (n = 16) purchased from Charles River (Wilmington, MA) were housed in a temperature-controlled room (23°C) with a 12:12-hour light–dark cycle. Half of the pregnant dams (n = 8) were assigned to the Alcohol group and the other half (n = 8) were individually paired to an Alcohol rat of similar weight and were assigned to the Pair-fed control group. All dams received a once-daily orogastric gavage and their feed intake was measured daily. The Alcohol group dams were acclimatized, via 4.5 g/kg ethanol (22.5% wt/v; peak blood alcohol concentration BAC, 216 mg/dl) from gestation day (GD) 5–10, followed by 6 g/kg ethanol from GD 11–20 or 21 (28.5% wt/v; peak BAC 289 mg/dl) (Davis-Anderson et al., 2018; Subramanian et al., 2014). The exposure protocol utilized in this study is based on established binge alcohol exposure paradigms across animal models and on reported alcohol consumption patterns in pregnant women (Caetano et al., 2006; Cudd et al., 2002; May et al., 2013; Ryan et al., 2008; Thomas et al., 2010). To control for nutrition from food and alcohol, the Pair-fed group dams were given the same amount of food as their paired-Alcohol group dams consumed, and received a once-daily maltose dextrin (50% wt/v) gavage, which was iso-caloric to the daily gavage amount of their paired Alcohol group dams. All animals were euthanized under isoflurane anesthesia.

Tissue Collection

On GD 20, in a subset of 8 dams (Pair-fed control, n = 4; alcohol, n = 4), two hours after respective administration of alcohol or maltose dextrin, maternal blood and fetal blood was collected in Ethylenediaminetetraacetic acid (EDTA) coated tubes. Fetal blood was collected from randomly selected pups and was pooled (segregated by sex) within each litter. Five aliquots (50 µl) of blood were immediately transferred to 6mm “punches” on blood spot

cards (Whatman 903 Protein Saver Card, GE Healthcare ltd), allowed to dry completely, and stored at room temperature (23°C), away from heat and light, until analysis. Maternal uterine arteries were excised, washed in HEPES–bicarbonate solution, pH 7.4 (NaCl 130 mM; KCl 4 mM; MgSO₄ · 7H₂O 2.5 mM; NaHCO₃ 4.05 mM; CaCl₂, 2.4 mM; HEPES 10 mM; KH₂PO₄ 1.18 mM; glucose 6 mM; EDTA 0.024 mM), weighed, immediately flash frozen, and stored at –80°C until analysis. In a separate set of 8 dams (Pair-fed control, n = 4; alcohol, n = 4), on GD 21, fetal brains were isolated in ice-cold ACSF buffer, pH 7.4 (NaCl 124 mM; KCl 2 mM; MgSO₄ · 7H₂O 2 mM; NaHCO₃ 4.05 mM; CaCl₂, 2 mM; KH₂PO₄ 1.25 mM; glucose 10 mM; oxygenated with a 95% O₂ and 5% CO₂ mixture), from a randomly selected male and female pup for hippocampal isolation. Utilizing the same isolation protocol, fetal cerebral cortex and cerebellum were isolated from 3 dams per treatment group. For collection, all fetal brains were cleaned of meninges, each region was isolated, weighed, and immediately frozen before storing at –80°C until analysis.

Measurement of PEth using LC/MS

Maternal and fetal blood: Each blood spot card was assayed by punching out the five blood spots for each sample (6mm diameter) into 16×100 polypropylene tubes. Subsequently, the internal standard (10 µl) followed by 2-propanol (2 ml) was added to each sample tube. Samples were briefly vortexed, shaken for 30 minutes, and were centrifuged for 30 minutes (x 3200 g), after which the supernatant was transferred to clean tubes and was dried under a nitrogen stream. The samples were then re-suspended in 100 µl of 1:1 buffers A and B (A: 40% 2mM ammonium acetate, 60% acetonitrile; B: 100% 2-propanol), micro-filtered, and transferred to injection vials. Ten microliters of each sample was injected into the LC/MS system for analysis (Biological Psychiatry Analytical Lab, University of Texas-Health Science Center, San Antonio, TX).

Uterine arteries and fetal brain regions: All other tissue samples (uterine artery, hippocampus, cerebral cortex, and cerebellum) were individually weighed and a 10X volume of 75% Methanol (MeOH) was added to each sample. For example, if the sample weighed 10 mg, a 100 µl volume of 75% MeOH was added to this. Each sample was then homogenized and 100 µl of sample homogenate was transferred to a clean tube. Internal standard (10 µl) was then added to the tissue homogenates, and these were vortexed for 20 seconds and centrifuged at 3200 g for 30 minutes. The supernatant was transferred into injection vials and 10 µl of supernatant was injected as described above onto the LC/MS system for analysis.

Data Analysis

Since PEth was not anticipated to be detected in the Pair-fed control group, the design included only the Alcohol group for statistical analyses. The data from male and female pups were comparable and no significant difference was detected, and hence these columns were collapsed. Normality was assessed using Shapiro-Wilk test. All blood PEth data were expressed as log-ng/ml of blood. Uterine artery and brain PEth data were expressed as log-pg/mg of tissue. A two-way ANOVA or a one-way ANOVA was utilized, where appropriate, and analysis was conducted similar to earlier studies (Thompson et al., 2016). Further, pairwise comparisons were done using Fisher's protected least significance difference

(PLSD). All values are expressed as mean $\pm$ SEM. Significance was established *a priori* at $P < 0.05$.

Results

As expected in the Pair-fed control group, both maternal and fetal blood PEth levels were below the detectable range (< 1.0 ng/ml) (Figure 2). Similarly, in the maternal uterine artery and fetal brain regions (hippocampus, cerebral cortex, and cerebellum), PEth was not detectable in the Pair-fed control group (< 0.1 ng/mg). In accordance with our hypothesis, all PEth homologues identified by LC/MS were detected in all tissue samples from the maternal and fetal compartments in the alcohol-administered treatment group.

PEth homologue distribution in blood of alcohol-administered group

There was a significant interaction between maternal/fetal blood PEth concentrations and the abundance of each of the three PEth isoforms (two-way ANOVA, Maternal/fetal compartment X PEth isoforms; $P = 0.0022$). In the maternal blood of alcohol-administered dams, the PEth 16:0/18:2 homologue concentration was 66% higher than that of PEth 16:0/18:1 ($P < 0.0001$), and was approximately 25% higher than that of PEth 16:0/20:4 ($P = 0.0072$). Also in the blood of alcohol-administered dams, PEth 16:0/20:4 was approximately 42% higher than PEth 16:0/18:1 ($P = 0.0015$). In the fetal blood from the alcohol-administered group, the PEth 16:0/18:2 homologue concentration was 20.4% higher than that of PEth 16:0/18:1 ($P = 0.0159$), and was approximately 19.4 % higher than that of PEth 16:0/20:4 ($P = 0.0187$). There was no difference between concentrations of the PEth homologues 16:0/18:1 and 16:0/20:4 in fetal blood. Interestingly, the PEth 16:0/18:2 and 16:0/20:4 homologue concentrations were approximately 25% ($P = 0.0020$) and 20% ($P = 0.0461$) higher, respectively, in maternal blood compared to fetal blood, whereas, there was no difference in the concentration level of PEth16:0/18:1 in maternal vs. fetal blood (Figure 3).

PEth homologue distribution in maternal reproductive and fetal brain regions

Overall, there was no difference in the concentrations among PEth homologues 16:0/18:1, 16:0/18:2, and 16:0/20:4 in the uterine artery (Figure 4). There was a significant interaction between fetal brain region PEth concentrations and of the three PEth isoforms (two-way ANOVA, fetal brain regions X PEth isoforms; $P = 0.0225$). In the fetal hippocampus, cerebral cortex, and cerebellum from the alcohol-administered group, the PEth homologue 16:0/18:1 concentration was significantly higher ($P < 0.0001$) than those of PEth 16:0/18:2 (~48–78 %) and PEth 16:0/20:4 (~31–62%), depending on brain region. PEth 16:0/20:4 was approximately 18% higher than PEth 16:0/18:1 ($P < 0.05$) in both the fetal hippocampus and cortex, but there was no difference between PEth 16:0/18:2 and 16:0/20:4 homologue concentrations in the fetal cerebellum. There was no difference in the PEth 16:0/18:1 concentration among the brain regions. PEth 16:0/18:2 and PEth 16:0/20:4 concentrations were significantly lower ($P < 0.05$) in the fetal cerebellum compared to the hippocampus and cortex (Figure 5).

Discussion

The current study aimed to quantify the PEth homologues 16:0/18:1, 16:0/18:2, and 16:0/20:4, and profile their distribution in maternal and fetal compartments following chronic gestational binge alcohol exposure. The results of this study can be summarized as follows: following an established chronic gestational binge alcohol paradigm: 1) all major PEth homologues were detected in the maternal and fetal blood; 2) particular isoforms, PEth 16:0/18:2 and 16:0/20:4, were significantly higher in the maternal blood compared to fetal blood, 3) all three PEth homologues were present in the uterine artery, which supplies oxygen and nutrients for fetal development; 4) all three PEth homologues were present in three fetal brain regions (hippocampus, cerebral cortex, and cerebellum) which are recognized as highly vulnerable to developmental alcohol exposure; and 5) the PEth isoform 16:0/18:1 was significantly higher than 16:0/18:2 and 16:0/20:4, in all three studied fetal brain regions. This study aligns with previous studies showing stability of PEth in maternal and fetal dried blood samples (Bakhireva et al., 2014; Baldwin et al., 2015).

PEth homologue concentrations in maternal and fetal blood

This is the first study to quantify three major PEth homologues, 16:0/18:1, 16:0/18:2, and 16:0/20:4 in both maternal and fetal blood following gestational binge alcohol exposure. In the Alcohol group, PEth 16:0/18:2 abundance was significantly higher than 16:0/18:1 and 16:0/20:4 in the maternal blood. Although, this is the first study to profile alcohol-induced PEth homologue distribution in pregnancy, a comparable pattern (16:0/18:2 > 16:0/18:1 > 16:0/20:4) was observed by Helander and Zheng in blood analyzed from a non-pregnant heavy drinker (Helander and Zheng, 2009). Similarly, a recent study in participants undergoing rehabilitation and withdrawal treatment observed that patients undergoing a relapse had a higher comparative increase in levels of PEth 16:0/18:2 to the levels of PEth 16:0/18:1 (Luginbühl et al., 2019). Previously, a study by Gnann and colleagues (Gnann et al., 2014), reported PEth 16:0/18:1 to be the most prominent homologue formed over a time period of 19 days in blood analyzed from patients undergoing withdrawal therapy. However, the study did not quantify PEth homologues, and instead compared the absolute peak area of PEth homologues over 19 days. The Gnann and colleagues' study relied on participant self-reporting, and as a result, the time of blood collection since last alcohol exposure were reported to be inconsistent. Interestingly, blood samples from a few self-identified social drinkers in this study also showed blood PEth 16:0/18:2 levels to be higher than PEth 16:0/18:1. In the current study, PEth 16:0/18:2 and 16:0/20:4 levels in maternal blood were significantly higher than in the fetal blood, with no difference in maternal PEth 16:0/18:1 concentration compared to their respective fetal blood concentrations. Since PEth homologues mirror their parent phosphatidylcholine homologues, the PEth 16:0/18:2 levels observed in the current study are consistent with the maternal and fetal blood PC 16:0/18:2 levels previously reported during pregnancy, wherein the PC 16:0/18:2 homologue was more abundant in maternal blood compared to the fetal blood (Postle, 1995).

Differences in PEth homologue levels can be attributed to the differential pharmacokinetic profiles of the three PEth homologues (Gnann et al., 2014; Javors et al., 2016; Lopez-Cruzan et al., 2018; Luginbühl et al., 2019). Among these, PEth 16:0/18:2 has the highest rate of

synthesis immediately following alcohol exposure, but shows a gradual concentration decrease over the subsequent period of abstinence (half-life: ~4–6 days) (Gnann et al., 2014; Luginbühl et al., 2019). PEth 16:0/18:1's rate of synthesis is reported to be slower than 16:0/18:2 but faster than 16:0/20:4, and is also the most stable among the three isoforms (half-life: ~8 days) (Luginbühl et al., 2019). PEth 16:0/20:4 has the slowest synthesis rate and the shortest half-life (half-life: ~2.1 days) (Lopez-Cruzan et al., 2018) of all three PEth homologues quantified in the current study. Considering these variances in synthesis and elimination rates of different PEth homologues, it may be possible to determine the amount, pattern, and timing of alcohol exposure based on their respective distribution profiles. Although the current study was performed under controlled conditions, studies have shown nutrition may have an effect on PC (PEth precursor) levels. For example, PC levels in the cerebral cortex of infants were altered depending on whether diet was breast milk- vs artificial formula-fed (Farquharson et al., 1995), and dietary fat has been shown to play a role in alcohol-induced mitochondrial PC levels (Thompson and Reitz, 1978). Although not extensively studied, some have suggested dietary preference may be a contributing factor in differential levels of alcohol-induced PEth homologue formation (Ulwelling and Smith, 2018; Viel et al., 2012).

PEth homologues distribution in the maternal primary uterine artery

This is the first study to quantify PEth levels in the maternal uterine artery, the vessel primarily responsible for delivering essential nutrients and oxygen to the developing fetus throughout pregnancy. Previous studies have demonstrated in both rat and sheep models that gestational alcohol exposure leads to uterine artery dysfunction, and that this alcohol-induced dysfunction may play an important role in FASD etiology (Gundogan et al., 2008; Naik et al., 2018; Ramadoss and Magness, 2012; Subramanian et al., 2014). Detection of all three major PEth homologues in the maternal uterine artery shows accumulation of PEth in the uterine arterial wall. Our data is in alignment with previous studies utilizing chronic and acute alcohol exposure paradigms (3 – 10g/kg/day) that have demonstrated PEth formation and accumulation in various organs such as the small intestines, stomach, spleen, heart, kidney, etc., of non-pregnant rats following alcohol exposure (Alling et al., 1984; Aradottir et al., 2002). PEth accumulation has been reported to disrupt lipid bilayer calcium-induced membrane fusion, as well as disrupt membrane bound protein function (Bondeson and Sundler, 1987; Omodeo-Salé et al., 1991; Rottenberg et al., 1992). Thus, it remains to be determined if accumulation of PEth in the uterine artery has an effect on the vessel function.

PEth profile in the fetal rat brain following gestational alcohol exposure

To our knowledge, this is the first study to quantify PEth homologues in the fetal brain. Additionally, we are the first to report a differential distribution of PEth homologues in fetal brain regions, i.e. the hippocampus, cerebral cortex, and cerebellum. These brain regions have been well documented as exquisitely vulnerable targets of developmental alcohol exposure (Berman and Hannigan, 2000; Jones and Smith, 1973; Lebel et al., 2011; Lunde-Young et al., 2018; Riley et al., 2004). In the current study, PEth 16:0/18:1 was ~48–78% higher than 16:0/18:2 and ~31–62% higher than 16:0/20:4 in all three regions. Although not in the fetal brain, a similar observation of PEth homologue distribution was found in the cerebellum and orbital frontal cortex in the post-mortem brain of adult humans diagnosed

with alcohol-use disorder, where PEth 16:0/18:1 levels were ~10 fold higher than PEth 16:0/18:2 levels (Thompson et al., 2016). Our data also aligns with other studies describing PEth formation in the adult rat brain following alcohol exposure (Kobayashi and Kanfer, 1987; Lundqvist et al., 1994). These differences in PEth homologue levels are consistent with the PC levels observed in the brain, for example, PC 16:0/18:1 is more abundant in the brain compared to 16:0/18:2 and 16:0/20:4 (Green and Yavin, 1996; Martínez and Mougán, 1998). PEth has been reported to have direct effects on various functionalities of the brain lipid bi-layer, such as the functional activity of membrane-bound enzymes and on inositol trisphosphate (IP3) receptor binding in the brain (Bondeson and Sundler, 1987; Lundqvist et al., 1994, 1993; Omodeo-Salé et al., 1991; Rottenberg et al., 1992). The fetal brain is a major organ of interest in discerning FASD etiology and our data provide additional evidence elucidating the direct and localized effects of alcohol on this vulnerable organ. Further research is warranted to understand the relationship between PEth distribution in the fetal brain and its plausible association(s) with neurodevelopmental/neurobehavioral effects of gestational alcohol exposure.

Overall, this study provides further evidence to investigate PEth as an ideal biomarker for detecting prenatal alcohol exposure. This study elucidates distribution of individual PEth homologue levels (16:0/18:1, 16:0/18:2, and 16:0/20:4) in the maternal blood, as a good indicator of alcohol exposure during pregnancy, although further studies are required to assess the effects of dose, timing, and detection window of the PEth homologue profiles. This study also provides insights into alcohol-induced PEth accumulation on critical FASD targets, specifically the maternal uterine artery and fetal brain. Future studies understanding functional impacts of gestational alcohol exposure-induced PEth accumulation in vital organs undergoing development are warranted.

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Figure 1: Structure of PEth homologues.

A) PEth 16:0/18:1, B) PEth 16:0/18:2, and C) PEth 16:0/20:4 isoforms, identified based on number of carbon and double bonds.

Figure 2: Example chromatogram of PEth 16:0/18:1 from fetal blood.

Representative chromatograms for A) PEth 16:0/18:1 analytical standard, B) PEth 16:0/18:1 calibrator (1000 ng/ml), C) Pair-fed control group, and D) Alcohol treatment group.

Figure 3: PEth homologue distribution in maternal and fetal blood following gestational alcohol exposure:

Concentrations of the PEth homologues 16:0/18:1, 16:2/18:2, and 16:0/20:4 were measured in maternal (Mat) and fetal blood (Fet). PEth was not detected (ND) in maternal or fetal blood of the Pair-fed control group (PF-Cont). There was a significant interaction between maternal/fetal compartments and the three PEth isoforms (two-way ANOVA, Maternal/fetal compartment X PEth isoforms; $P = 0.0022$). In the maternal blood of alcohol administered dams, PEth 16:0/18:2 was significantly higher than both PEth 16:0/18:1 (*, $P < 0.05$) and 16:0/20:4 (*, $P < 0.05$). PEth 16:0/20:4 was significantly higher than PEth 16:0/18:1 (*, $P < 0.05$). PEth 16:0/18:2 and PEth 16:0/20:4 concentrations were significantly higher in maternal blood compared to that of fetal blood (*, $P < 0.05$) in the Alcohol group. Data expressed as mean $\pm$ SEM. Significance was established *a priori* at $P < 0.05$.

Figure 4: PEth homologue distribution in maternal uterine artery following gestational alcohol exposure:

Concentrations of the PEth homologues 16:0/18:1, 16:2/18:2, and 16:0/20:4 were measured in the maternal uterine artery. PEth was not detected (ND) in the uterine arteries of the Pair-fed control group (PF-Cont). All three PEth homologues were detected in the maternal uterine arteries of the alcohol-administered animals (Alcohol). Overall, there was no difference in concentrations among PEth homologues 16:0/18:1, 16:0/18:2, and 16:0/20:4 in the uterine artery. Data expressed as mean $\pm$ SEM.

Figure 5: PEth homologue distribution in fetal brain regions following gestational alcohol exposure:

Concentrations of the PEth homologues 16:0/18:1, 16:2/18:2, and 16:0/20:4 were measured in the fetal hippocampus (Hippo), cerebral cortex (Cortex), and cerebellum (Cereb). PEth was not detected (ND) in the fetal brain regions of the Pair-fed control group (PF-Cont). There was a significant interaction between fetal brain region PEth concentrations and abundance of each of the three PEth isoforms (two-way ANOVA, fetal brain regions X PEth isoforms; $P = 0.0225$). ‘*’ represents significance difference between PEth homologue concentrations (*, $P < 0.05$). Data expressed as mean $\pm$ SEM. Significance was established *a priori* at $P < 0.05$.