EFFECTS OF MATERNAL L-GLUTAMINE SUPPLEMENTATION ON FETUS TO MITIGATE TERATOGENIC EFFECTS OF ALCOHOL

A Dissertation

by

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ABSTRACT

Women who drink alcohol during pregnancy are at high risk of giving birth to children with physical, behavioral or cognitive developmental problems called Fetal Alcohol Spectrum Disorders (FASD). Prenatal alcohol exposure is known to be associated with fetal growth restriction, disturbances in amino acid bioavailability, and alterations in fetal hemodynamics, blood flow and oxidative stress. Alterations in these parameters can persist into adolescence and low birth weight can lead to altered fetal development and programming, which can have lifelong consequences. Glutamine has been associated with fetal nitrogen and carbon metabolism, synthesis of the cellular anti-oxidant glutathione, apoptosis suppression, serving as a precursor for the synthesis of other amino acids, and increases in protein synthesis. Glutamine has been used clinically as a nutrient supplement in low birth weight infants. Therefore, it is hypothesized that repeated third trimester-equivalent maternal alcohol exposure in the sheep model decreases the bioavailability of amino acids, hampers fetal body growth, alters maternal-fetal hemodynamics, hampers uterine blood flow, alters fetal blood flow, increases cerebellar oxidative stress and that maternal L-glutamine supplementation may attenuate these negative developmental effects of prenatal alcohol exposure.

Maternal alcohol exposure during the third trimester-equivalent period in the sheep model significantly reduced fetal body weight, height, crown-rump length and thoracic girth, and maternal glutamine supplementation successfully improved these fetal growth parameters in the alcohol+glutamine group. Maternal alcohol exposure during the third trimester-equivalent period resulted in significant reduction in glutamine

and glutamine related amino acids bioavailability in maternal and fetal plasma as well as in the fetal amniotic and allantoic fluid. Maternal glutamine supplementation improved the bioavailability and efficacy of amino acids in the maternal and fetal compartment. This study also revealed that maternal alcohol exposure resulted in maternal acidemia, maternal hypercapnea, maternal hypoxemia as well as fetal acidemia and fetal hypercapnea, but not fetal hypoxemia. Maternal alcohol exposure during this period led to an increase in fetal mean arterial pressure, alterations in fetal brain blood flow and fetal cerebellar oxidative stress. Maternal alcohol exposure during the third trimester-equivalent period resulted in a more than 40% reduction in uterine artery blood flow. Maternal glutamine supplementation during the third trimester-equivalent period successfully attenuated the incidences of alcohol-induced maternal hypercapnea, fetal acidemia, alterations in fetal brain blood flow and improved the fetal cerebellar endogenous antioxidant status. Collectively these results signify that maternal glutamine supplementation mitigates negative developmental effects of prenatal alcohol exposure.

DEDICATION

To my parents

&

Karmanye Vadhikaraste, Ma phaleshou kada chana,

Ma Karma Phala Hetur Bhurmatey Sangostva Akarmani

BHAGAVAD GITA 2.47

In simple terms it could mean: Keep on performing your duties without thinking of results and eventually your actions will be rewarded

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1. INTRODUCTION AND BACKGROUND

1.1. Teratogenic effects of alcohol

Maternal alcohol exposure can have detrimental effects on the cognitive and behavioral function of a child. Neurophysiological studies have identified deficits in learning, memory and functioning in children with fetal alcohol spectrum disorders (FASD). FASD is an umbrella term encompassing the full range of effects that can occur in an individual whose mother consumed alcohol during pregnancy. These effects on physical, behavioral or cognitive development can persist as lifelong disabilities, with the most severe end of the spectrum being Fetal Alcohol Syndrome (FAS) [1-4]. The term FAS was first coined by Jones and Smith in 1973 to describe a pattern of birth defects observed in children of mother who consumed alcohol during pregnancy [5]. A distinctive pattern of facial abnormalities, growth deficits manifested by low birth weight, small height and head size (microcephaly), and central nervous system abnormalities are the three primary defining diagnostic features of FAS [6]. However, not all children prenatally exposed to alcohol exhibit the characteristic facial features of FAS. These children, who essentially do not meet all of the diagnostic criteria of FAS are referred to as having partial FAS (pFAS), fetal alcohol effects (FAE) or alcohol-related neurodevelopmental disorders (ARND) [7].

1.2. Prevalence of FASD

Despite the knowledge on the devastating effects of alcohol consumption during pregnancy on a child, and in spite of massive educational efforts via various multi-

institutional campaigns to educate women about the adverse effect of alcohol consumption during pregnancy, the incidence of maternal alcohol consumption has not gone down significantly. As per recent reports by the substance abuse and mental health services administration (SAMHSA), 9.4% of pregnant women were involved in alcohol drinking during pregnancy in 2011 and this number was increased from 10% in 2009 to 10.8% in 2010. It is very important to note that 55.1% women of child bearing age (aged 15-44) in the United States are still involved in alcohol drinking, hence potentially increasing the risk of FASD in the future [8-10]. As per recent reports, 2-7 cases of FAS per 1000 children are estimated and all levels of FASD may be around 2-5% of total children [11, 12].

1.3. Mechanisms of alcohol-induced damage, role of acidosis and glutamine

Identifying the one mechanism or exact cellular and molecular pathway contributing to alcohol-induced developmental damage is very difficult because alcohol exhibits coexistence of a multiple and simultaneous mode of actions among various cell types. Increased oxidative stress, mitochondrial damage, alteration in cell-cell adhesion, impairments in growth factors related signaling pathways and DNA damage are some of the mechanisms by which alcohol executes developmental damage [13]. Interestingly, even before Fetal Alcohol Syndrome was first described by Jones and coworkers [5], scientists suggested fetal acidosis as a potential mechanism for the teratogenic effects of alcohol [14]. Alcohol exposure in human clinical cases is known to result in a mixed respiratory and metabolic acidosis [15-17] and in animal models, acidemia has been described as a candidate mechanism for alcohol-induced developmental neuronal injury,

bone growth retardation and altered amino acid homeostasis [18-21]. Previous studies have shown that single or repeated alcohol exposure or acidemia during the third trimester-equivalent period in sheep results in a decrease in maternal and fetal glutamine bioavailability [19, 22]. Glutamine plays a crucial role in pH homeostasis through its metabolism in the kidney [23-25]. The kidneys are capable of extracting and catabolizing more than one third of plasma glutamine in a single pass under acidic conditions [26]. Alcohol-induced acidemia stimulated glutamine uptake by renal mitochondria and its metabolism via phosphate-dependent glutaminase (PDG) pathway [27] could be the key mechanism contributing to a decrease in plasma glutamine in response to alcohol-induced acidemia [19, 22]. Phosphate-dependent kidney glutaminase utilizes glutamine as a substrate and gives stoichiometric amounts of glutamate and ammonia [28]. Ammonium ions are mainly excreted in the urine to facilitate the excretion of metabolic acids or anions while conserving sodium and potassium ions, hence contributing to counteract the acidosis [29].

1.4. Purpose of this work

Based on earlier findings, it is evident that prenatal alcohol exposure leads to alterations in amino acids homeostasis. The fetus depends on steady supply of nutrients for growth and development, and disturbances in this supply can lead to impaired fetal development and growth retardation. A number of amino acids, especially glutamine and glutamine related amino acids have been demonstrated to be reduced in the maternal and fetal compartments in response to gestational alcohol exposure in rodent and sheep models [19, 22, 30-33]. Previously, glutamine was classified as a nonessential amino

acid but due to its multifaceted role in cell signaling, survival and growth, it is now referred to as a conditionally essential dietary amino acid. Diminished glutamine uptake and alterations in glutamine and glutamine-dependent metabolic processes lead to significant modifications of physiological and immunological functions [34]. This work will explore an intervention utilizing the pluripotent amino acid, glutamine to counteract negative developmental effect of prenatal alcohol exposure. Chapter 2 examines the effect of maternal alcohol exposure and glutamine supplementation during the third trimester-equivalent period on fetal growth and amino acids bioavailability in fetal plasma, amniotic fluid and allantoic fluid. Chapter 3 evaluates the effect of alcohol exposure and glutamine supplementation on maternal-fetal hemodynamics, uterine perfusion and fetal regional brain blood flow. Chapter 4 highlights the role of oxidative stress in alcohol-induced fetal cerebellar damage and the effect of maternal glutamine supplementation on the cellular endogenous antioxidant system. All these studies are done during the third trimester-equivalent period in the sheep model.

1.5. Significance of the sheep model

The sheep model has been widely used in research to understand human reproductive biology. The sheep model possesses major advantages for the study of FAS when compared with other species. First, the adult ewe and fetus are tolerant of handling, surgery and instrumentation, features that facilitate longer duration treatment and blood sampling. A second major advantage of the sheep is the large body mass. The sheep fetus weighs between 0.85-4.50 kg during the third trimester equivalent (this weight range represents the rapid growth during this period of gestation), while the

adults sheep weighs between 55-85 kg, comparable with that of a human fetus and an adult woman respectively. The relatively large blood volume of sheep allows the simultaneous measurement of multiple dependent variables at multiple time points. Such experiments are relatively very difficult to perform in small laboratory animals. A third major advantage of sheep is that this species has a much longer length of gestation, which allows intervening or performing experiments at specific times during gestation. A fourth and most important advantage is that the all three trimester-equivalents of human pregnancy and peak velocity of brain growth, called the "brain growth spurt" occur *in-utero* in the sheep, whereas it occurs postnatally in rodent models [35, 36]. The first 6 weeks of sheep gestation is roughly equivalent to the first trimester in humans, weeks 7 to 13 are equivalent to the second trimester and weeks 14 to 19.5 encompass the third trimester equivalent of human brain growth-spurt.

2. EFFECTS OF MATERNAL GLUTAMINE SUPPLEMENTATION ON ALCOHOL-INDUCED FETAL GROWTH RESTRICTION AND AMINO ACID BIOAVAILABILITY

2.1. Introduction

In children, prenatal alcohol exposure has been associated with fetal growth deficits [37-39]. Widespread work by Day and colleagues has shown that growth deficits arising from prenatal alcohol exposure can persist into adolescence [40-46]. Animal studies, particularly using the rodent and sheep model have shown that alcohol exposure can produces growth deficits and impaired skeletal development in the offspring [21, 47-52]. As per the Barker hypothesis low birth weight can lead to altered development and programming by increasing the risk of cardiovascular disorders later in life [53]. Although fetal growth deficits as a diagnostic feature has received minimal attention compared to the facial and CNS abnormalities, more attention should be given to the alcohol-induced fetal growth deficits since it has been associated with other prenatal and neonatal anomalies. For example, low birth weight has been associated with childhood mortality and morbidity [54, 55], lower I.Q. and learning disabilities [56, 57], sleep disturbances [58], hyperactivity [59], delayed reflex and motor disturbances [60], hypertension [61], metabolic diseases [62, 63], osteoporosis [64, 65], respiratory issues [66] and mental disorders such as schizophrenia [67, 68].

The fetus depends on steady supply of nutrients for growth and development, and disturbances in this supply can lead to impaired fetal development and growth restriction. A number of amino acids have been demonstrated to be reduced in the

maternal and fetal compartments in response to gestational alcohol exposure in rodents [30-33]. Previously, a study from our lab reported that a chronic third trimester-equivalent alcohol exposure results in decreases in maternal glutamine and glutamine-related amino acid levels [19]. Glutamine is a conditionally essential amino acid and it is involved in many vital cellular processes [69-73]. Glutamine supplementation in many animal studies and in clinical cases has been shown to improve growth function and efficacy of amino acids [74-79]. In another study from our lab, we have shown that an acute alcohol exposure during the third trimester-equivalent period results in a simultaneous decrease in maternal and fetal glutamine and glutamine-related amino acids, and administration of single aqueous bolus of L-glutamine improved the amino acids profile in the maternal as well as the fetal compartment [22]. However, the effect of repeated alcohol exposure and concurrent maternal L-glutamine supplementation during the third trimester-equivalent period on fetal growth and amino acid bioavailability has not been studied before. Therefore, we hypothesized that repeated third trimester-equivalent alcohol exposure reduces the bioavailability of amino acids in the fetal compartment and hampers fetal body growth, and that concurrent maternal L-glutamine supplementation will improve the bioavailability of amino acids and mitigate alcohol-induced growth deficits.

2.2. Materials and methods

2.2.1. Animals

All aspects of the surgical and experimental protocols were approved by the Texas A&M University Institutional Animal Care and Use Committee. Suffolk ewes aged 2-5 years were obtained from a commercial supplier. Upon arrival at the animal facility, each ewe received an intramuscular injection of Covexin® 8 (Merck Animal Health, Summit, NJ) and an oral bolus of Valbazen® (Zoetis, Kalamazoo, MI). Ewes received progesterone impregnated vaginal implants (EAZI-BREED™, CIDR®, Zoetis, Kalamazoo, MI). Implants were removed 11 days after placement at which time prostaglandin $F_{2\alpha}$ (20 mg; LUTALYSE®, Zoetis, Kalamazoo, MI) was intramuscularly administered. The following day, ewes were placed with a ram fitted with a marking harness for a period of 24 h. Marked ewes were presumed pregnant until confirmed pregnant ultrasonographically on gestation day (GD) 25 [52].

Upon confirmation of pregnancy, ewes were housed individually where they were able to have visual contact with herd mates in adjacent pens at all times. Conditions of constant temperature (22 ^{0}C) and fixed light dark cycle (12:12) were maintained. Ewes were fed TAMU Ewe Ration, a custom ration (Nutrena, Cargill Animal Nutrition, Minneapolis, MN), twice daily. Ration amount was based on body weight and determined by ARIES® software version 2007 (University of California, Davis). This was a pelleted feed consisting of minimum 12.75% protein, minimum 3.00% crude fat, and maximum 25.00% crude fiber. Two different dosing paradigms were used in this study and they are depicted in Figure 1. Animals from experiment 1 (Figure 1A)

received treatment from GD 109 to 132, and animals from experiment 2 (Figure 1B) received treatment from GD 99 to 120. Ewes from experiment 2 were allowed ad libitum access to water but underwent an 18 h fasting period before the surgical procedure on GD 117±1; food was withheld before the start of the final infusion on GD 120±1 for experiment 2 animals and on GD 132 for experiment 1 animals. Daily feed consumption was monitored; subjects in all the treatment groups consumed all of the food offered.

2.2.2. Treatment groups

Four treatment groups were used in this study for experiment 1 and 2: 1. saline control group that received 0.9% saline (N=15 and 10), 2. an alcohol group that received alcohol at a dosage of 2.5 g/kg body weight (40% w/v diluted in 0.9% saline) (N=17 and 8), 3. Glutamine group that received 0.9% saline and 100 mg/kg of L-glutamine 3 times a day, this group will serve as the control group for glutamine supplementation (N=16 and 10), and 4. An alcohol+glutamine group that received alcohol at a dosage of 2.5 g/kg body weight (40% w/v diluted in 0.9% saline) and 100 mg/kg of L-glutamine 3 times a day (N=17 and 12). The alcohol dose of 2.5 g/kg was selected to generate blood alcohol concentration in the range of 300-370 mg/dl to mimic a binge alcohol exposure producing pathological fetal cerebellar Purkinje cell loss [80]. The ewes in the glutamine and alcohol+glutamine groups received glutamine at the dose of 100 mg/kg of body weight 3 times per day. No adverse effects or safety concerns of IV or oral glutamine supplementation were observed in newborns or in adult humans for glutamine doses in the range of 400-860 mg/kg/day [81]. In these studies, safety assessments were done by evaluating standard clinical chemistry, mental status, vital signs, temperature and clinical

and subjective evidence of toxicity [81]. Due to the short half-life of glutamine [82] and for the ease of administration, glutamine supplementation was done 3 times per day. Detailed description of the alcohol and glutamine dosing paradigm is given in the next section.

2.2.3. Dosing paradigm

Two different dosing paradigms were used in this study and they are depicted in Figure 1. Animals from experiment 1 (Figure 1A) received treatment from GD 109 to 132, and animals from experiment 2 (Figure 1B) received treatment from GD 99 to 120.

For experiment 1 animals, alcohol or saline infusions were given intravenously (IV) through a jugular vein catheter over 1 hour from GD 109 to 132, 3 consecutive days per week to mimic a weekend binge drinking pattern. This period of gestation in sheep overlaps with the human third trimester-equivalent brain growth spurt [35, 36]. On gestational day 109, an intravenous catheter (16 gauge, 3.00 in Extended Use Catheter, Jorgensen, Loveland, CO) was placed percutaneously into the jugular vein. On the days of infusions, ewes were connected to the infusion pump by 0830 h and alcohol or saline was infused continuously over 1 h. Infusion solutions were delivered intravenously by peristaltic pump (VetFlo® 7701B IV Vet Infusion Pump, Grady Medical, Temecula, CA). The first four doses of alcohol were 1.75, 2, 2.25 and 2.25 g/kg respectively and thereafter were 2.5 g/kg (figure 1). The saline control and glutamine groups received a dose of 0.9% saline that was isovolumetric to the alcohol groups. L-Glutamine powder from Sigma Aldrich (Cat #5792) was completely dissolved in sterile water at a concentration of 4.5% w/v and passed through a 0.2 μm bacteriostatic filter. The solution

was kept at room temperature and prepared no sooner than 1 to 2 h prior to administration. A 100 mg/kg dose of glutamine was administered IV as a 4.5% w/v aqueous bolus three times a day on 3 consecutive days per week. On GD 132±1 animals received either saline or alcohol infusion as described earlier. Animals from glutamine and alcohol+glutamine groups received a single bolus of glutamine (4.5% w/v, 100 mg/kg) just before the start of final infusion. Maternal blood alcohol concentration at the end of infusion on GD 132 was estimated using an enzymatic assay kit (Quantichrom® ethanol assay kit; BioAssay Systems, Hayward, CA). At the end of 60 min, the ewes were euthanized using an IV injection of sodium pentobarbitone (75 mg/kg). The uterus was removed from the ewe and the fetus was exteriorized. The fetus was removed after carefully collecting fetal amniotic and allantoic samples. Fetal blood was collected quickly before measuring fetal body weight, abdominal and thoracic girth, crown-rump length, height, head length, head width and head circumference.

For experiment 2 animals, a similar procedure was followed from GD 99 to 115 and ewes underwent surgery on GD 117±1.

2.2.4. Surgical procedure

For experiment 2, surgical instrumentation and catheterization of the ewe and fetus were performed on GD 117±1. Surgery was conducted using sterile technique and all components were cold-gas sterilized using an AN-74*i* tabletop sterilizer (Andersen Products, Haw River, NC). Surgical anesthesia was induced with intravenous ketamine hydrochloride 6.0 mg/kg (Ketaved™, Vedco, St. Joseph, MO) and diazepam 0.3 mg/kg (Abbott Labs, Abbott Park, IL) and the trachea intubated. Surgical anesthesia was

maintained with 2-3% isoflurane (Fluriso, VetOne, Boise, Idaho) in oxygen delivered by a ventilation system (Matrx™ Model 3000, Midmark, Orchard Park, NY), and heart rate, ventilation rate, oxygen saturation, and expired carbon dioxide were monitored throughout the surgery using a Datascope Passport® 2 monitor (Mindray, Mahwah, NJ). Ewes were placed lying in the dorsal position throughout the surgery. Using standard surgical techniques, catheters were inserted into the left and right maternal femoral artery and vein and both the left and right fetal cranial tibial arteries and fetal saphenous veins and were advanced to the abdominal aorta and inferior vena cava, respectively [20]. The fetus was then returned to the uterus. The catheters were filled with heparinized saline and sealed. All the catheters were tunneled subcutaneously and then exteriorized through a small incision in the right flank of the ewe.

Upon completion of the surgery (1.5-2 h) and post-operative recovery (1 h), the ewe returned to her pen. Buprenorphine hydrochloride (0.3 mg) (Buprenex®, Reckitt Benckiser, Berkshire, UK) was administered intramuscularly & flunixin meglumine 1.1 mg/kg (Banamine®, Merck Animal Health, Summit, NJ) was administered orally to the ewe every 12 h to control postoperative pain. In addition to procaine G penicillin 20,000 IU/kg (PenOne Pro, VetOne, Boise, Idaho) prophylactic antibiotic therapy included 3 doses of gentamicin sulfate (2.0 mg/kg) (VetOne, Boise, Idaho) intramuscularly. All ewes were allowed to recover completely from instrumentation before exposing to the final treatment and conducting the experiment on GD 120±1.

On GD 120±1 animals received either saline or alcohol infusion as described earlier. Baseline (0 min) maternal and fetal arterial blood was collected, and after that

animals from glutamine and alcohol+glutamine group received single bolus of 4.5% w/v (100 mg/kg) of glutamine just before the start of final infusion. Maternal and fetal arterial blood was collected at the end of infusion (60 min). Maternal blood alcohol concentration at the end of infusion on GD 120±1 was estimated using an enzymatic assay kit (Quantichrom® ethanol assay kit; BioAssay Systems, Hayward, CA). At the end of 60 min, the ewes were euthanized using an IV injection of sodium pentobarbitone (75 mg/kg). The uterus was removed from the ewe and the fetus was removed. Fetal body weight, abdominal and thoracic girth, crown-rump length, height, head length, head width and head circumference were measured.

2.2.5. Amino acid analysis

50 µL of fetal plasma, amniotic and allantoic fluid from experiment 1 and 0-60 min maternal and fetal plasma from experiment 2 were acidified with 50 µL of 1.5 mM $HClO_4$ and then neutralized with 20 µL of 2 mM K_2CO_3. 900 µL of water was added to this solution and samples were centrifuged at 10,000 rpm for 5 min. The supernatant fluid was used for amino acid analysis by HPLC, as described previously [83]. Concentrations of amino acids in samples were quantified on the basis of authentic standards from Sigma Chemicals (St. Louis, MO, USA) using the Waters Millenium-32 workstation (Waters Corporation, Milford, MA).

2.2.6. Statistical analysis

Two-way mixed ANOVA was performed for the analysis of fetal growth parameters with treatment group and number of fetuses (single, twin or triplet) as

factors. One-way ANOVA was performed for the analysis of amino acid levels among treatment groups. Further pairwise comparisons were performed when appropriate using Fisher's protected least significant difference. Level of significance was established at $P<0.05$ and $0.05<P<0.1$ were considered trends.

2.3. Results

2.3.1. Blood alcohol concentrations

The mean ± SEM maternal blood alcohol concentrations (BACs) at the end of final alcohol infusion (60 min; the time point when BACs are known to peak) on GD 132±1 were 313.56±15.93 mg⁄dl and 308.84±13.40 mg⁄dl in the alcohol and alcohol+glutamine treatment groups, respectively. BACs at the end of alcohol infusion on GD 120±1 were 304.20±27.19 mg⁄dl and 325.40±24.93 mg⁄dl in the alcohol and alcohol+glutamine treatment groups, respectively. The differences between the alcohol and alcohol+glutamine treatment groups BAC were not statistically significant.

2.3.2. Fetal growth parameters

Fetal body weight ($F(3,86)=3.007$, $P=0.035$), crown-rump length ($F(3,85)=4.587$, $P=0.006$) and height ($F(3,84)=3.278$, $P=0.025$) exhibited significant main effect of treatment groups. Fetuses from the alcohol group had significantly lower body weight, height, crown-rump length, thoracic girth and head width compared to the saline control group (all $P\leq0.001$) (Table 1). Fetal body weight ($P<0.001$), height ($P=0.002$), crown-rump length ($P=0.006$) and thoracic girth ($P=0.004$) in the alcohol+glutamine group were significantly higher than the alcohol group, indicating that maternal glutamine

supplementation attenuated alcohol-induced fetal growth deficits. No significant interaction between treatment group and number of fetuses was observed for any of the dependent parameters, except for fetal head width ($F(5,85)=3.594$, $P=0.005$). Maternal weight on GD 132 was not significantly different among groups. Mean ± SEM of growth parameters on GD 132 and on GD 120±1 and details about statistically significant differences are tabulated in Table 1 and 2, respectively.

2.3.3. Fetal plasma amino acid concentrations on GD 132

Amino acid concentrations in fetal plasma were significantly altered among groups for asparagine, glutamine, histidine and threonine. Concentrations of asparagine and histidine in fetal plasma were significantly decreased in the alcohol group compared to the saline control ($P=0.021$ and 0.008, respectively) and glutamine ($P=0.002$ and 0.016, respectively) groups. Concentration of glutamine in fetal plasma was significantly decreased in the alcohol groups compared to the saline control ($P=0.026$), glutamine ($P<0.001$) and alcohol+glutamine ($P=0.014$) groups. Concentration of threonine in fetal plasma was significantly decreased in the alcohol and alcohol+glutamine groups compared to the saline control group ($P=0.003$ and 0.037, respectively). Mean ± SEM of Fetal plasma amino acid levels on GD 132 and details about statistically significant differences are tabulated in Table 3.

2.3.4. Fetal amniotic fluid amino acid concentrations on GD 132

Amino acid concentrations in fetal amniotic fluid were significantly altered among groups for asparagine, serine, glutamine, threonine, citrulline, tyrosine and

leucine. Concentration of asparagine in fetal amniotic fluid was significantly decreased in the alcohol group compared to the saline control (P=0.011) and alcohol+glutamine (P=0.018) groups and showed a decreasing trend compared to the glutamine group (P=0.094). Concentration of serine in fetal amniotic fluid was significantly decreased in the alcohol group compared to the glutamine (P=0.015) and alcohol+glutamine (P=0.011) groups. Concentration of glutamine in fetal amniotic fluid was significantly decreased in the alcohol group compared to the saline control (P=0.016) and glutamine (P=0.017) groups and showed a decreasing trend compared to the alcohol+glutamine group (P=0.092). Concentration of glycine in fetal amniotic fluid was significantly decreased in the alcohol group compared to the saline control (P=0.025) and alcohol+glutamine (P=0.033) groups. Concentration of threonine in fetal amniotic fluid was significantly decreased in the alcohol group compared to the saline control group (P=0.001). Concentrations of citrulline and leucine in fetal amniotic fluid were significantly decreased in the alcohol and glutamine groups compared to the saline control group (all $P<0.05$). Concentration of alanine in fetal amniotic fluid was significantly decreased in the alcohol group compared to the saline control (P=0.034) and glutamine (P=0.017) groups and showed a decreasing trend compared to the alcohol+glutamine group (P=0.071). Concentration of tyrosine in fetal amniotic fluid was significantly increased in the alcohol+glutamine group compared to the alcohol (P=0.019) and glutamine (P=0.006) groups. Mean ± SEM of fetal amniotic fluid amino acid levels on GD 132 and details about statistically significant differences are tabulated in Table 4.

2.3.5. Fetal allantoic fluid amino acid concentrations on GD 132

Amino acid concentrations in fetal amniotic fluid were significantly altered among groups for asparagine, threonine, taurine, tyrosine and tryptophan. Concentration of asparagine in fetal allantoic fluid was significantly decreased in the alcohol group compared to the saline control (P=0.013) and glutamine (P=0.012) groups. Concentration of histidine in fetal allantoic fluid was significantly decreased in the alcohol group compared to the saline control group (P=0.020) and exhibited a decreasing trend compared to the glutamine (P=0.078) group. Concentration of threonine in fetal allantoic fluid was significantly decreased in the alcohol and alcohol+glutamine group compared to the saline control group (P<0.001 and =0.003, respectively). Concentration of arginine in fetal allantoic fluid was significantly decreased in the alcohol and alcohol+glutamine groups compared to the glutamine group (P=0.020 and 0.038, respectively). Concentration of taurine in fetal allantoic fluid was significantly decreased in the alcohol group compared to the saline control (P=0.008) and glutamine (P=0.028) groups, and it was significantly decreased in the alcohol+glutamine group compared to the saline control group (P=0.031). Concentration of tyrosine in fetal allantoic fluid was significantly decreased in the alcohol group compared to the glutamine group (P=0.008). Concentration of tryptophan in fetal allantoic fluid was significantly decreased in the alcohol and alcohol+glutamine groups compared to the glutamine group (P=0.006 and 0.009, respectively). Mean ± SEM of fetal allantoic fluid amino acid levels on GD 132 and details about statistically significant differences are tabulated in Table 5.

2.3.6. Acute changes in maternal plasma amino acids on GD 120±1.

Statistically significant differences were observed in the percent change in maternal arterial plasma amino acid concentration from baseline (0 min) to the end of infusion (60 min) among groups for the following amino acids: aspartate, glutamate, asparagine, glutamine, citrulline, arginine and taurine. Figure 2 highlights the amino acids in which changes between baselines (0 min) and end of infusion (60 min) were significantly different between groups. The percent changes in all the amino acid concentrations with the details of which groups were statistically different for each amino acid are summarized in Table 6.

2.3.7. Acute changes in fetal plasma amino acids on GD 120±1.

Statistically significant differences were observed in the percent change in fetal arterial plasma amino acid concentration from baseline (0 min) to the end of infusion (60 min) among groups for the following amino acids: glutamate, asparagine, serine, glutamine, glycine, threonine, citrulline, valine, isoleucine, leucine, ornithine and lysine. Figure 3 highlights the amino acids in which changes between baselines (0 min) and end of infusion (60 min) were significantly different between groups. The percent changes in all the amino acid concentrations with the details of which groups were statistically different for each amino acid are summarized in Table 7.

2.4. Discussion

Four vital findings can be gleaned from this study; first, maternal alcohol exposure during the third trimester-equivalent period causes intra uterine growth

restriction (IUGR). Second, maternal glutamine supplementation during the third trimester-equivalent period was able to prevent alcohol-induced IUGR. Third, maternal alcohol exposure leads to significant alterations in amino acid bioavailability in the maternal and fetal compartments. Fourth, maternal glutamine supplementation during the third trimester-equivalent period was able to improve fetal amino acids bioavailability.

2.4.1. Alcohol exposure hampers fetal growth

Our finding shows that maternal alcohol exposure during pregnancy hampers fetal growth, and this was evident by decreases in fetal body weight, height, crown-rump length, thoracic girth and head width. Clinical studies conducted by various investigators at different locations report that women who consumed alcohol during pregnancy gave birth to the fetuses with lower birth weight and length, smaller head and chest circumference [84-88]. Day and colleagues evaluated the long term effects of prenatal alcohol exposure on growth in adolescence. They assessed fetal growth at birth, at 8 and 18 months, and at 3, 6, 10 and 14 years of age. They identified that growth deficits associated with prenatal alcohol exposure still persistent in offspring at the age of 14 and their weight, height, head circumference and skin thickness was significantly affected [40, 42, 43, 45, 46, 89]. In another study, investigators reported that children who were exposed to alcohol during pregnancy had smaller head circumferences at the age of 5-8 years [90].

Animal studies have also reported that developmental alcohol exposure leads to growth deficits. Chronic low to moderate maternal alcohol consumption (6% v/v, 15%

derived calories) during pregnancy in Sprague-Dawley rats resulted in significant decrease in fetal body weight and hind limb length on embryonic day 20 and significant decrease in snout–rump length and crown–rump length was observed at 8 months of age compared to the control group [50]. Moderate to heavy maternal alcohol exposure (20-35% derived calories) in the rodent model during pregnancy has been shown to decrease birth weight and size [51, 91-94]. These results support our findings that prenatal alcohol exposure results in fetal growth deficits.

2.4.2. Importance of amino acids

Amino acids play crucial role in maintaining normal physiological function and nutritional status of the body [95, 96]. Amino acids that regulate the key metabolic pathways of cell survival, growth, development, and reproduction of animals have recently been introduced as "functional amino acids" [97, 98]. The term "functional amino acids" encompasses arginine, cysteine, glutamine, leucine, and proline which are known to improve the efficiency of utilization of dietary proteins [74, 99, 100]. The main function of dietary amino acids is to stimulate muscle protein synthesis. Individual amino acids have been implicated directly or indirectly in immune function [100], and some are important precursors of neurotransmitters and certain hormones [98, 101, 102]. Sufficient bioavailability of amino acids in the fetal compartment is not only required for fetal development, but also essential to reduce the risk of chronic diseases in adult life [103].

2.4.3. Alcohol alters amino acid bioavailability

Results from this study indicate that repeated maternal alcohol exposure during the third trimester-equivalent period in the sheep model significantly decreased the bioavailability of asparagine, glutamine, histidine and threonine in the fetal plasma and asparagine, glutamine, glycine, threonine, citrulline, alanine and leucine in the fetal amniotic fluid. Levels of asparagine, histidine, threonine, taurine and ornithine were reduced in the fetal allantoic fluid. During gestation, the fetus is suspended in the amniotic fluid compartment, which is a significant source of fetal nutrients and connected to the allantoic sac via the urachus. Allantoic fluid plays a vital role in accumulation and transfer of nutrients [69]. A number of amino acids have been demonstrated to be reduced in the maternal and fetal compartments in response to gestational alcohol exposure in rodents. Acute alcohol exposure (0.03 ml/g, 25% v/v) in the pregnant mouse model resulted in a significant reduction in plasma concentrations of threonine, serine, glutamine, glycine, alanine, and methionine [30]. Chronic alcohol exposure during the first two trimester-equivalents of human brain growth [31] modeled in the rat has been shown to reduce maternal plasma proline and fetal plasma aspartate concentrations [104]. Utilizing a chronic alcohol exposure (6% v/v, 36% derived calories) paradigm during the first two trimester-equivalents of human brain growth (until 20 days of rat gestation), Karl and coworkers found elevation in plasma glutamate in the fetal rat, but not in the mother [105]. The current report demonstrates that in maternal plasma glutamine, asparagine and arginine were significantly decreased due to alcohol exposure and glutamine supplementation improved the concentrations of these

amino acids. Previously, a study from our lab reported that chronic third trimester-equivalent alcohol exposure (1.75 g/kg) in a weekend binge drinking pattern (from GD 109-132) resulted in a decrease in glutamine and glutamate, and an increase in methionine, leucine, valine and overall branched-chain amino acids (BCAA) in maternal plasma. In this study [19] the authors also reported that the final alcohol exposure on GD 132 (which followed the chronic alcohol exposure) resulted in a decrease in arginine, asparagine, citrulline, threonine, tryptophan, methionine, leucine, histidine, tyrosine, valine and isoleucine levels in maternal plasma [19]. In another study from our lab, we showed that a single alcohol exposure (1.75 g/kg) during the third trimester-equivalent period resulted in a decrease in glutamine, citrulline, BCAA, serine and asparagine in maternal plasma, and glutamine, phenylalanine, asparagine and tryptophan in fetal plasma [22]. Collectively these reports support our findings and imply that alcohol exposure during pregnancy alters amino acid bioavailability in the maternal and fetal compartments.

Acute maternal alcohol exposure on GD 120±1, which was followed by the repeated maternal alcohol exposure during the third trimester-equivalent period significantly increased the level of glutamate in maternal and fetal plasma at the end of infusion compared to the baseline. This finding of increased levels of glutamate after alcohol exposure is consistent with earlier findings [19, 22, 33]. Alcohol-induced acidemia [21] leads to the activation of phosphate dependent glutaminase in the kidney [106], which utilizes glutamine as a substrate and gives stoichiometric amounts of glutamate and ammonia, hence reducing glutamine bioavailability [28]. This mechanism

is supported by the findings that acidemia reduces plasma glutamine levels in nonpregnant sheep, humans, and rats [107-109]. It is also important to note that corticosteroid administration in humans to mimic stress conditions has been demonstrated to increase glutamine uptake in the splanchnic bed and could thus explain the decrease in glutamine bioavailability under stress conditions [110]; earlier findings in the sheep and rodent models and children suggest that alcohol exposure increases both maternal and fetal glucocorticoid levels [111-115].

2.4.4. Role of glutamine in metabolism

Glutamine is an abundant free amino acid in plasma and cells and it is an intermediate member of many metabolic pathways and acts as a signaling molecule. Glutamine is the most abundant amino acid in the blood and the free amino acid pool in the body [116]. The bioavailability of glutamine in ovine fetal plasma is 2-3 times greater than that of maternal plasma, between GD 60-140 [69]. The fetal liver hydrolyses glutamine to release glutamate into the fetal systemic circulation and fetal the placenta extensively metabolizes glutamate to yield glutamine [73, 117]. Earlier glutamine has been classified as a nonessential amino acid but due to its multifaceted role in cell signaling, survival and growth, it is now classified as a conditionally essential dietary amino acid. Diminishing glutamine uptake and alterations in glutamine and glutamine-dependent metabolic processes leads to significant modifications of physiological and immunological functions [34]. Glutamine is known to play a crucial role in the transamination, inter-organ metabolism of nitrogen and carbon as well as serving as the primary oxidative fuel for enterocytes and lymphocytes [73, 116, 118]. Glutamine is an

important precursor of the brain neurotransmitter glutamate, cellular anti-oxidant glutathione, nucleotides and other macromolecules [69, 70, 72]. Glutamine has also been associated with having an important role as an apoptosis suppressor [119].

2.4.5. Effect of glutamine supplementation on amino acid bioavailability and growth

Glutamine is an important precursor and mediator for the synthesis of many amino acids including glutamate, arginine, proline, asparagine, ornithine and citrulline [71, 120]. Maternal glutamine supplementation during the third trimester-equivalent period in sheep improved the amino acid profiles in the maternal as well as fetal compartments [22]. The findings that glutamine supplementation decreased the levels of BCAA (leucine, isoleucine and valine) in fetal plasma could indicate an overall increase in protein synthesis and decrease in protein wasting. Similar to our results, a decrease in plasma leucine concentration has been reported after enteral glutamine administration [121, 122] and a decrease in BCAA was observed after alanine-glutamine infusion in rats [123]. Glutamine supplementation in postweaning pigs prevented jejunal atrophy, increased plasma concentration of aspartate, glutamate and alanine, improved body weight gain, increased intestinal expression of genes related to the cell growth and antioxidants, and suppressed expression of genes that promote oxidative stress and immune activation [74, 76]. A recent study has shown that dietary supplementation with up to 4% mono-sodium glutamate is safe and improved growth performance in postweaning pigs [124]. Haynes and colleagues reported that glutamine administration in neonatal piglets enhanced growth performance and prevented endotoxin-induced enterocytes death by reducing intestinal expression of Toll-like receptor-4, active

caspase-3 and NF-κB [125]. Glutamine supplementation in severely ill or extremely low birth weight infants has shown improvement in physical growth, neurodevelopmental outcomes, hepatic tolerance, plasma glutamine concentrations and lowered infectious morbidity [75, 77-79]. These results from human clinical cases and animal studies support our findings that maternal glutamine supplementation mitigate alcohol-induced fetal growth deficits and improves amino acid bioavailability.

In summary, results from this chapter support the conclusion that repeated alcohol exposure during the third trimester-equivalent period in sheep alters amino acid homeostasis and maternal glutamine supplementation attenuated this effect. These findings may help understand mechanisms behind alcohol-induced IUGR and neurodevelopmental damage. Positive effects of maternal glutamine supplementation form the basis for use of glutamine to counteract the negative effects of prenatal alcohol exposure, which we will be discussed in the following chapters.

3. EFFECTS OF GESTATIONAL ALCOHOL EXPOSURE AND L-GLUTAMINE SUPPLEMENTATION ON MATERNAL AND FETAL HEMODYNAMICS, UTERINE BLOOD FLOW, AND FETAL REGIONAL BLOOD FLOW

3.1. Introduction

Alcohol is a neurotoxic teratogen and many studies have shown that alcohol exposure has the potential to cause damage in different regions of the developing brain [1, 126-130]. Although the deleterious effects of maternal alcohol exposure on fetal brain development and function are well documented, little is known about its effects on maternal and fetal cardiovascular adaptations [131].

Alcohol is reported to produce dose-dependent changes in maternal and fetal systemic hemodynamics in animal models. For instance, in sheep, lower doses of alcohol (0.75 g/kg) do not result in any change in fetal mean arterial pressure (MAP) or heart rate (HR), whereas higher doses result in altered systemic hemodynamics [20, 132]. These alterations were accompanied by alcohol-induced maternal/fetal acidemia, hypercapnea and hypoxemia [20, 21, 132, 133]. In the rat model, prenatal alcohol exposure (35% alcohol-derived calories) during the final one-third of pregnancy had no effect on HR in the adult offspring [134]. Ultrasound imaging studies have shown that binge like alcohol (3 g/kg) exposure in a murine second trimester equivalent model does not alter fetal HR [135].

Among the maternal cardiovascular changes during normal pregnancy, the most important are the vascular adaptations of the uterine artery that supplies nutrients and

oxygen to the feto-placental compartment. Dramatic increases in uterine blood flow occur over the course of normal pregnancy to accommodate the increasing needs of nutrients and oxygen to the developing fetus and placenta [131]. These adaptations include a decrease in uterine vascular resistance, increase in angiogenesis and extracellular matrix remodeling leading to a 53-fold increase in uterine blood flow compared to the non-pregnant state [136-139]. Therefore, a decrease in uterine perfusion is a cardinal feature of intrauterine growth restriction. Alterations in uterine blood flow can be closely associated with fetal cardiovascular adaptation and can predispose the fetus to cardiovascular diseases in the future by altering vascular reactivity [140, 141]. Unfortunately, the impact of maternal alcohol exposure on uterine perfusion has received minimal attention and there is a paucity of information. An earlier study in sheep has shown that acute alcohol exposure leads to a significant reduction in uterine blood flow [142] and series of *in-vitro* studies have shown that binge alcohol exposure alters uterine vascular adaptations [143-147]. However, to our knowledge, so far no study has been done to investigate the effect of repeated alcohol exposure during pregnancy on uterine blood flow. Therefore, this study will thoroughly investigate the effect of repeated binge maternal alcohol exposure on uterine artery blood flow during the third trimester-equivalent period.

Studies performed using a sheep model have shown that gestational alcohol exposure alters fetal brain blood flow and vascular reactivity [132, 148-151]. Our lab [152] and others [149] have shown that fetal brain blood flow increases in response to acute alcohol exposure in sheep. Cerebral vasodilatory response to hypoxemia was

significantly attenuated in neonatal lambs exposed to alcohol during the first trimester-equivalent period of gestation [148]. Ultrasound imaging studies have shown that binge like alcohol exposure in a murine second trimester equivalent model results in rapid and persistent decrease in blood flow from the umbilical artery to the fetal brain [135]. Although collectively these studies imply that alcohol exposure leads to an alteration in fetal brain blood flow, no study has been done to investigate the effect of an intervention strategy to restore alcohol-induced alterations in brain blood flow.

The effect of L-glutamine was investigated as a nutrient intervention to mitigate the alcohol-induced alterations in maternal-uterine-placental-fetal blood flow, based on the findings that repeated alcohol exposure or acidemia during the third trimester-equivalent period results in a decrease in maternal and fetal glutamine bioavailability [19, 22]. Research has shown that glutamine plays a crucial role in pH homeostasis through its metabolism in the kidney [23-25]. Two diverse effects of L-glutamine on vascular reactivity have been observed. *In-vivo* and *in-vitro* studies have shown that L-glutamine influences endothelial-dependent relaxation by inhibiting availability of NADPH [153] and by inhibiting recycling of citrulline to arginine to limit nitric oxide release [154-160], whereas some other studies have postulated that a longer duration vasodilatory effect of glutamine is by the donation of glutamine derived nitrogen atom for nitric oxide or other vasodilator synthesis [161]. Thus, we hypothesized that repeated third trimester-equivalent maternal alcohol exposure would lead to maternal and fetal acidemia-hypercapnea and alter systemic hemodynamic parameters. We also hypothesized that alcohol exposure would decrease uterine blood flow. We further

hypothesized that alterations in these parameters would lead to subsequent changes in fetal regional brain and peripheral blood flow, and glutamine supplementation would attenuate these negative effects of alcohol.

3.2. Materials and methods

3.2.1. Animals

As explained earlier in the section 2.2.1.

3.2.2. Treatment groups

As explained earlier in the section 2.2.2.

The number of animals in each group was 10, 8, 10 and 12 for the saline control, alcohol, glutamine and alcohol+glutamine groups, respectively.

3.2.3. Dosing paradigm

Alcohol or saline infusions were given intravenously (IV) through a jugular vein catheter over a period of one hour from GD 99 to 115 on 3 consecutive days per week to mimic a weekend binge drinking pattern. This period of gestation in sheep overlaps with the human third trimester-equivalent brain growth spurt [35, 36]. On gestational day 99, an intravenous catheter (16 gauge, 3.00 in Extended Use Catheter, Jorgensen, Loveland, CO) was placed percutaneously into the jugular vein. On the day of infusions, ewes were connected to the infusion pump by 0830 h and alcohol or saline was infused continuously over one h. Infusion solutions were delivered intravenously by peristaltic pump (VetFlo® 7701B IV Vet Infusion Pump, Grady Medical, Temecula, CA). The first

four doses of alcohol were 1.75, 2, 2.25 and 2.25 g/kg on GD 99, 100, 101 and 106 respectively and thereafter were 2.5 g/kg from GD 107 (Figure 1, Panel B). The saline control and glutamine groups received a dose of 0.9% saline that was isovolumetric to the alcohol groups. L-Glutamine powder from Sigma Aldrich (Cat #5792) was completely dissolved in sterile water at a concentration of 4.5% w/v and passed through a 0.2 μm bacteriostatic filter. The solution was kept at room temperature and prepared no sooner than 1 to 2 h prior to administration. A 100 mg/kg dose of L-glutamine was administered IV as a 4.5% w/v aqueous bolus three times a day on 3 consecutive days per week. On GD 120±1, animals received either saline or alcohol infusion as described in earlier. Animals from glutamine and alcohol+glutamine groups received a single bolus of glutamine (100 mg/kg, 4.5% w/v) just before the start of the final infusion.

3.2.4. Surgical procedure

Surgical instrumentation and catheterization of the ewe and fetus were performed on GD 117±1. Surgery was conducted using sterile technique and all components were cold-gas sterilized using an AN-74*i* tabletop sterilizer (Andersen Products, Haw River, NC). Surgical anesthesia was induced with intravenous ketamine hydrochloride 6.0 mg/kg (Ketaved™, Vedco, St. Joseph, MO) and diazepam 0.3 mg/kg (Abbott Labs, Abbott Park, IL) and the trachea intubated. Surgical anesthesia was maintained with 2-3% isoflurane (Fluriso, VetOne, Boise, Idaho) in oxygen delivered by a ventilation system (Matrx™ Model 3000, Midmark, Orchard Park, NY), and heart rate, ventilation rate, oxygen saturation, and expired carbon dioxide were monitored throughout the surgery using a Datascope Passport® 2 monitor (Mindray, Mahwah, NJ). Ewes were

placed lying in the dorsal position throughout the surgery. Using standard surgical techniques [20], catheters were inserted into the left and right maternal femoral artery and vein and both the left and right fetal cranial tibial arteries and saphenous veins and were advanced to the abdominal aorta and inferior vena cava, respectively. The fetus was then returned to the uterus. A 6 mm transient-time ultrasonic perivascular flow probe (Transonic Systems Incorporated, Ithaca, NY, USA) was secured around the primary uterine artery for recording uterine blood flow (UBF). As the incisions were closed, a catheter was installed inside the amniotic cavity. The catheters were filled with heparinized saline and sealed. All the catheters and flow probe leads were tunneled subcutaneously and then exteriorized through a small incision in the right flank of the ewe.

Upon completion of the surgery (1.5-2 h) and post-operative recovery (1 hour), the ewe returned to her pen. Buprenorphine hydrochloride (0.3 mg) (Buprenex®, Reckitt Benckiser, Berkshire, UK) was administered intramuscularly and flunixin meglumine 1.1 mg/kg (Banamine®, Merck Animal Health, Summit, NJ) was administered orally to the ewe every 12 h to control postoperative pain. In addition to penicillin G procaine 20,000 IU/kg (PenOne Pro™, VetOne, Boise, Idaho), prophylactic antibiotic therapy included 3 doses of gentamicin sulfate (2.0 mg/kg) (VetOne, Boise, Idaho) intramuscularly. All ewes were allowed to recover completely from instrumentation before exposing to the final treatment and conducting the experiment on GD 120±1.

3.2.5. Measurement of systemic hemodynamic variables and uterine blood flow

On GD 120±1, by 0830 hour ewes were transferred to a specialized cage where lateral and linear movement was restricted. One of the maternal arterial, fetal arterial and the amniotic catheters were connected to a strain-gauge pressure transducer and phasic blood pressure was sampled at 1000Hz continuously throughout the experiment. Uterine artery blood flow recording from Transonic TS420 Perivascular Flowmeter and blood pressure recording was collected using a PowerLab® data acquisition system (PowerLab 8/30, model ML870) and was analyzed using LabChart® software (ADInstruments, Inc., Colorado Springs, CO). Maternal and fetal arterial blood was collected at the baseline (0 min) and at the end of infusion (60 min) for the analysis of arterial pH, partial pressure of CO_2 (PCO_2), bicarbonate (HCO_3) and partial pressure of O_2 (PO_2) using i-Stat portable clinical analyzer (model 300A) (Abbott, Inc., Princeton, NJ). Maternal blood alcohol concentration at the end of infusion (60 min) was measured using an enzymatic assay kit (Quantichrom® ethanol assay kit; BioAssay Systems, Hayward, CA).

3.2.6. Measurement of fetal regional blood flow

Fetal regional blood flow measurements were done using stable non-radioactive labeled microsphere technique [162]. Stable gold labeled microspheres of 15 μm in diameter (BioPAL Inc., Worcester, MA), suspended in normal saline containing tween 80 and 0.01% thimerosal at a concentration of 2500000 spheres/ml were injected as a 1-1.5 mL bolus into the fetal inferior vena cava via the saphenous vein catheter at the baseline (0 min). The reference blood samples were withdrawn from the fetal abdominal aorta via the cranial-tibial artery catheter by precision syringe pump (Harvard Apparatus,

Holliston, MA, USA) at a constant speed of 2.06 ml/min. A similar procedure was repeated at the end of the infusion (60 min) using samarium labeled microspheres. Readers need to note that for the practicality of surgical procedures and experimentation, reference blood was drawn from the fetal abdominal aorta. Although reference blood drawn from the fetal abdominal aorta would not allow the measurement of actual fetal brain blood flow since distribution of blood between ascending and descending aorta is different [163], it will allow estimation of relative change in regional blood flow at the end of 60 min compared to the baseline. Therefore, fold changes in fetal regional blood flows are reported and not the absolute values. At the end of 60 min, ewes were euthanized using an IV injection of sodium pentobarbitone (75 mg/kg). The uterus was removed from the ewe and the fetus was exteriorized. The brain was removed from the fetus and the rest of the fetus was dissected to obtain the organ/tissue samples. The brain was further dissected into 8 regions: frontal cortex, parietal cortex, temporal cortex, occipital cortex, cerebellum, brain stem, olfactory bulbs and hippocampus. During sample dissection, careful consideration was given to maintain accurate and precise sectioning for each animal. The samples were weighed and placed into specialized sample vials. Tissue and reference blood samples were dried overnight at 70 ^{0}C and shipped to Bio Physics Assay Laboratory (BioPAL Inc., Worcester, MA), where they were exposed to a field of neutrons. The sample vials were stored for 48 h to allow short-lived activation products to decay. The results of the assay were reported as the number of disintegrations per min (DPM) measured for each stable labeled microsphere. Detailed description of the neutron activation procedure is explained elsewhere [162].

Absolute tissue blood flow at different times, measured by stable labeled microspheres was calculated using following formula,

$$\text{Blood flow (ml/min/g)} = \frac{2.06 \text{ (ml/min)} \times [\text{Microspheres in tissue}]}{\text{Tissue weight (g)} \times [\text{Microspheres in reference blood}]}$$

where 2.06 mL/min is the reference blood withdrawal rate, [Microspheres in reference blood] is the DPMs in reference blood, tissue weight was in grams and [Microspheres in tissue] is the DPMs in the tissue sample. For ease of understanding and simplicity of data presentation, fold change in blood flow in any tissue was calculated by dividing blood flow at the end of infusion (60 min) by blood flow at the baseline (0 min).

3.2.7. Statistical analysis

Two-way mixed ANOVA was performed on the maternal and fetal systemic hemodynamic parameters at the baseline (0 min) and at the end of infusion (60 min) on GD 120±1 with treatment group and time point as factors. Mixed ANOVA was performed on the maternal uterine blood flow values for every 5 min time interval starting from the baseline (time 0) to the end of infusion (60 min) on GD 120±1 using treatment group and each time point as factors. Further pairwise comparisons were performed when appropriate using Fisher's protected least significant difference method. One-way ANOVA was performed among groups for the analysis of fold change in regional blood flow. Level of significance was established at $P<0.05$ and $0.05<P<0.1$ were considered trends.

3.3 Results

3.3.1. BAC

The mean ± SEM maternal BACs at the end of alcohol infusion (60 min; the time point when BACs are known to peak) on GD 120±1, were 304.20±27.19 mg⁄dl and 325.40±24.93 mg⁄dl in the alcohol and alcohol+glutamine treatment groups, respectively. The difference between the alcohol and alcohol+glutamine treatment groups BAC was not statistically significant.

3.3.2. Maternal systemic hemodynamic parameters

No changes were observed for maternal MAP and HR among groups at the baseline (0 min, i.e. the time when BAC is 0 mg/dl) or at the end of infusion (60 min) on GD 120±1 (Figure 4, Panel A and B). Baseline measurements on GD 120±1 depict the chronic effect of repeated alcohol exposures during the third trimester-equivalent period and measurements done at the end of infusion on GD 120±1 depict the effect at peak BAC. Baseline maternal arterial pH did not differ among groups, but at the end of infusion, maternal arterial pH was significantly decreased in the alcohol and alcohol+glutamine group compared to the saline control group, glutamine group and compared to their respective baseline (all $P<0.001$) (Figure 4, Panel C). Baseline maternal arterial PCO_2 was significantly higher in the alcohol group compared to the saline control ($P=0.008$), glutamine ($P=0.021$) and alcohol+glutamine ($P=0.022$) groups (Figure 4, Panel D). Maternal arterial PCO_2 was significantly elevated at the end of infusion in the alcohol and alcohol+glutamine groups compared to the saline control and

glutamine groups (all P<0.001). Maternal arterial PCO_2 was significantly elevated at the end of infusion in the alcohol+glutamine group compared to its baseline (P<0.001). Levels of maternal arterial bicarbonate (HCO_3) at the baseline did not differ among groups but were significantly decreased at the end of infusion in the alcohol group compared to the saline control (P=0.046) and glutamine (P=0.031) groups (Figure 4, Panel E). The level of maternal arterial bicarbonate (HCO_3) at the end of infusion in the alcohol group was significantly lower than its baseline (P=0.019) and the level of maternal arterial bicarbonate (HCO_3) at the end of infusion in the alcohol+glutamine group showed a decreasing trend compared to its baseline (P=0.095). Baseline maternal arterial PO_2 did not differ among groups, but at the end of infusion maternal arterial PO_2 was significantly decreased in the alcohol and alcohol+GLN group compared to the saline control group (P=0.003 and <0.001, respectively), glutamine group (all P<0.001) and compared to their respective baselines (Alcohol, P=0.006 and alcohol+glutamine, P=0.033) (Figure 4, Panel F). These results indicate that maternal alcohol exposure during the third trimester-equivalent period results in maternal acidemia, hypercapnea and hypoxemia and maternal glutamine supplementation was able to compensate for these acid-base imbalances from alcohol.

3.3.3. Fetal systemic hemodynamic parameters

In the alcohol group baseline (0 min) fetal MAP was significantly higher compared to the saline control group (P=0.039) and fetal MAP at the end of infusion (60 min) was significantly elevated compared to the saline control (P=0.006) and glutamine (P=0.025) groups on GD 120±1 (Figure 5, Panel A). No changes were observed for fetal

HR among groups at the baseline or at the end of infusion on GD 120±1 (Figure 5, Panel B). Baseline fetal arterial pH did not differ among groups, but at the end of infusion fetal arterial pH was significantly decreased in the alcohol and alcohol+GLN group compared to the saline control group ($P<0.001$ and $=0.002$, respectively), glutamine group (all $P<0.001$) and compared to their respective baselines (all $P<0.001$) (Figure 5, Panel C). Interestingly, fetal arterial pH at the end of infusion in the alcohol group was significantly lower than the alcohol+glutamine group ($P=0.049$). Baseline fetal arterial PCO_2 did not differ among groups and fetal arterial PCO_2 was significantly elevated at the end of infusion in the alcohol and alcohol+glutamine groups compared to the saline control group ($P<0.001$ and $=0.001$, respectively), glutamine group (all $P<0.001$) and compared to their respective baselines (Alcohol, $P=0.28$ and alcohol+glutamine, $P=0.003$) (Figure 5, Panel D). No changes were observed for fetal arterial bicarbonate (HCO_3) and PO_2 among groups at the baseline (0 min) or at the end of infusion (60 min) on GD 120±1 (Figure 5, Panel E and F). These results indicate that the maternal alcohol exposure during the third trimester-equivalent period leads to an increase in fetal MAP, acidemia and hypercapnea and that maternal glutamine supplementation during the third trimester-equivalent period was able to mitigate these effects of alcohol on the fetus**.**

3.3.4. Maternal uterine artery blood flow

A significant main effect of treatment group ($P<0.001$) on uterine blood flow was present, but no significant effect of time or no significant interaction between treatment group and time was noted. Uterine artery blood flow rate was significantly decreased in the alcohol and alcohol+glutamine groups compared to the saline control and glutamine

groups (all P<0.001) (Figure 6). No significant differences were observed between saline control group and glutamine group, as well as between alcohol group and alcohol+glutamine group. These results indicate that repeated maternal alcohol exposure during the third trimester-equivalent period decreases uterine artery blood flow and no significant effect of maternal glutamine supplementation on uterine artery blood flow was observed.

3.3.5. Fetal regional blood flow

A more than two fold increase was observed in the fetal cerebellar, brain stem and olfactory bulb blood flow at the end of 60 min from their respective baseline in the alcohol group (Figure 7, Panel A). Fold change in fetal cerebellar and brain stem blood flow in the alcohol group was significantly higher than the saline control (Cerebellum, P=0.002 and brain stem, P=0.001), glutamine (Cerebellum, P=0.011 and brain stem P=0.009) and alcohol+glutamine (Cerebellum, P=0.025 and brain stem, P=0.015) groups. Blood flow in the fetal olfactory bulb was significantly higher in the alcohol group than the saline control (P=0.012) and glutamine (P=0.025) groups. Further, the blood flow at the end of 60 min in the alcohol group exhibited a numerically increasing pattern in the hippocampus, frontal cortex, parietal cortex, temporal cortex and occipital cortex; however, the increases were not statistically significant. Estimated fetal whole-brain blood flow in the alcohol group was significantly higher than the saline control group (P=0.013) (Figure 7, Panel A). Blood flow to fetal placenta, liver, kidney, myocardium, skeletal muscles, skin and jejunum was not significantly different among groups (Figure 7, Panel B). These results indicate that maternal alcohol exposure results

in a transient increase in fetal brain blood flow, especially to the cerebellum, brain stem and olfactory bulb. Glutamine supplementation was able to prevent these alcohol-induced alterations in fetal regional blood flow.

3.4. Discussion

Five major findings can be gleaned from this study; first, maternal alcohol exposure leads to maternal acidemia, hypercapnea and hypoxemia. Second, maternal alcohol exposure results in an increase in fetal mean arterial pressure, acidemia and hypercapnea. A third and most important finding of this study is that maternal repeated binge alcohol exposure during the third trimester-equivalent period in the sheep model results in a more than 40% reduction in uterine artery blood flow. Fourth, maternal alcohol exposure leads to a transient increases in fetal brain blood flow, leading to increased brain alcohol delivery. The fifth and most innovative finding from this study is that maternal glutamine supplementation was able to diminish alcohol-induced maternal hypercapnea, fetal acidemia and alterations in fetal regional brain blood flow, hence limiting the brain-alcohol delivery during the periods of prenatal alcohol exposure.

3.4.1. Effect of alcohol on systemic hemodynamic parameters

Results from this study confirm the earlier findings [18, 20, 22, 132, 133] that alcohol exposure during the third trimester-equivalent period in sheep leads to maternal acidemia, hypercapnea and hypoxemia and fetal acidemia and hypercapnea but not fetal hypoxemia. Alcohol exposure in human clinical cases is known to result in mixed respiratory and metabolic acidosis [15-17] and in animal models, acidemia has been

described as a candidate mechanism for alcohol-induced developmental neuronal injury and altered amino acid homeostasis [18-20]. Although there is no direct data from human studies on the effect of maternal alcohol consumption during pregnancy on systemic hemodynamic parameters, many clinical studies in men and non-pregnant women have shown that alcohol consumption directly or indirectly contributes to the development of hypertension [164-168].

The absence of alterations in alcohol-induced maternal HR and MAP demonstrates that alcohol-induced decreases in uterine blood flow are not due to a change in the uterine perfusion resulting from systemic hemodynamics, but rather is a local effect of alcohol on the gestational uterine artery adaptations. In rats, prenatal alcohol exposure (35% alcohol derived calories) during the final one-third of pregnancy (from GD 14 to 20) had no effect on HR in the offspring as adults [134]. Ultrasound imaging studies have shown that binge like alcohol exposure (3 g/kg) in a murine second trimester equivalent model did not alter fetal HR [135]. Kenna and colleagues also reported that prenatal alcohol exposure (0.75 g/kg) during the third trimester-equivalent period (from GD 95 to 133) in sheep did not alter fetal HR [169]. These reports support our findings that repeated maternal alcohol exposure during the third trimester-equivalent period in sheep had no significant effect on fetal HR. It is important to note that there are differences among these studies in duration of exposure period and severity of alcohol exposure.

3.4.2. Alcohol exposure during third trimester-equivalent period impairs uterine blood flow

In the current study we observed approximately 40% reduction in uterine artery blood flow following repeated third trimester-equivalent repeated binge alcohol exposure. Our finding was strongly supported by an earlier acute alcohol exposure study in pregnant sheep during third trimester-equivalent period, where intravenous infusion of 1 g alcohol/min over 1 h decreased uterine as well as placental blood flow, and the reductions were maintained for at least 2 h after the end of alcohol treatment; uterine blood flow was significantly decreased by more than 20%, whereas the umbilical blood flow was significantly decreased by more than 30% [142]. On the contrary, another study in the ovine model has shown that acute alcohol infusion on GD 124±3 increases uterine artery blood flow in dose dependent manner [170]. Studies have shown that vasodilator nitric oxide (NO) production by activation of endothelial NO synthase (eNOS) is a major local regulator of uterine blood flow during pregnancy [171-174]. A study in pregnant C57BL/6J mice demonstrated that NO modulation of the systemic mesenteric artery vascular response was hampered due to alcohol exposure [175]. *In-vitro* studies have shown that binge-like alcohol exposure alters eNOS activation and reduces angiogenic mRNA gene expression and proteome in the ovine uterine artery endothelial cells, hence blunting the uterine vascular adaptations including vasodilatory and angiogenic pathways [131, 143, 145-147, 176]. These findings support our results about the deleterious effect of alcohol on uterine blood flow and potential mechanisms by which alcohol exposure impairs uterine blood flow needs to be elucidated.

3.4.3. Alcohol exposure alters fetal cerebral blood flow

We herein hypothesized that repeated alcohol exposure during the third trimester-equivalent period would alter fetal brain blood flow and that maternal glutamine supplementation would ameliorate this effect. An acute alcohol exposure on GD 120±1 resulted in a transient increase in fetal cerebellar, olfactory bulb, brain stem and overall whole-brain blood flow and maternal glutamine supplementation corrected this effect. Mann and co-workers have shown that fetal cerebral blood flow increases in response to acute alcohol exposure in sheep [149]. Alcohol exposure on GD 132, which was followed by the chronic maternal alcohol exposure during the third trimester-equivalent period in sheep (from GD 109 to 132) leads to increases in cerebral blood flow, especially in the ethanol-sensitive cerebellum [132]. Ultrasound imaging studies have shown that binge alcohol (3 g/kg) exposure in a murine second trimester equivalent model results in rapid and persistent decrease in blood flow from the umbilical artery to the fetal brain on GD 12.5-14.5; in this study blood flow was assessed by measuring fetal arterial blood acceleration and velocity time integral (VTI) data from pulse wave Doppler imaging experiments [135]. Alcohol (1.5 g/kg) exposure during the second trimester-equivalent period in sheep from GD 60-90 decreased and from GD 30-82 increased the subsequent cerebral vasodilatory responses to hypoxia and to vasodilatory hormones like vasoactive intestinal peptide, respectively [150, 151]. Gleason and colleagues demonstrated that the cerebral vasodilatory response to hypoxemia was significantly attenuated, showing that cerebral O_2 delivery was not maintained in 1-4 day old neonatal lambs exposed to the alcohol (1 g/kg) during the first trimester-equivalent

period of gestation [148]. In rats, moderate alcohol exposure (20 to 60 mmol/l) doesn't alter the reactivity of the cerebral pial arteriole but exposure to higher doses of alcohol (80 to 100 mmol/l) impairs the dilatory response to agonists that trigger the synthesis of NO from the endothelium and neurons [177]. Human studies performed using single photon emission computerized tomography technique revealed abnormalities and regional differences in cerebral blood flow in response to alcohol consumption and these differences were dependent on degree of intoxication and age [178-180]. These clinical and preclinical investigations support our findings and emphasize that alcohol exposure alters cerebral vascular regulation.

3.4.4. Effect of glutamine on acid-base balance

Results from our study demonstrate that maternal glutamine supplementation was able to diminish the alcohol-induced maternal hypercapnea, transient fetal acidemia and alterations in fetal regional brain blood flow. Alcohol-induced acidemia stimulated glutamine uptake by renal mitochondria and its metabolism via the phosphate-dependent glutaminase gives stoichiometric amounts of glutamate and ammonia [27, 28]. Ammonium ions are mainly excreted in the urine to facilitate the excretion of metabolic acids or anions while conserving sodium and potassium ions, hence contributing to counteraction of the acidosis [29]. Mitigation of alcohol induced-acidemia in the fetal but not in the maternal compartment could be attributed to the fact that the fetus is capable of extracting glutamine from the placenta and extracted glutamine is delivered into the fetal circulation at a rate that is the highest among all other amino acids [117, 181, 182]. Also, the bioavailability of glutamine in ovine fetal plasma is 2-3 times

greater than that of maternal plasma throughout gestation [69]. However, it should be noted that levels of maternal bicarbonate were significantly decreased in the alcohol group but not in the alcohol+glutamine group at the end of 60 min on GD 120±1. These findings indicates that maternal glutamine supplementation during the third trimester-equivalent period was able to correct alcohol-induced acid-base imbalances.

3.4.5. Role of acid-base imbalance and glutamine on vascular function

One study in a rat model showed that cerebrovascular reactivity in response to alcohol exposure is mediated by hypercapnea [183]. Studies in non-pregnant monkey, dog and perinatal goat models have demonstrated that cerebral blood flow was significantly increased in response to acute metabolic and respiratory acidosis, and this increase in cerebral flow was linearly related to the degree of hypercapnic acidemia [184-186]. Many studies have demonstrated the ability of glutamine to impair NO mediated vasodilation by inhibiting citrulline uptake and arginine synthesis or by metabolism of glutamine to glucosamine which in turn inhibits availability of NADPH (an essential cofactor for NOS) [153, 158, 159, 187, 188]. Therefore, all together these findings support our results and imply that alcohol-induced acidemia-hypercapnea is possibly a key mechanism underlying alcohol-induced increase in fetal cerebral blood flow and glutamine-induced decreases in NO release and the capability of glutamine to counteract acid-base imbalances may be responsible for preventing alcohol-induced increases in brain blood flow. It should be noted that we and others have shown that glutamine exhibits a gluconeogenic effect [22] via activation of glucagon secretion [189] and glucagon is known to increase blood flow [190-192]. Houdijk and colleagues have

reported that a glutamine-enriched enteral diet increased splanchnic blood flow independent of activation of glucagon and NO [193]. These findings indicate that the effect of glutamine on blood flow and vascular reactivity towards acidemia-hypercapnea differs temporally and regionally and probably contributes to the regional differences in alterations in blood flow.

In summary, results from this chapter imply that repeated maternal alcohol exposure in sheep during the third trimester-equivalent period results in maternal and fetal acidemia and hypercapnea, maternal but not fetal hypoxemia, elevation in fetal MAP, decrease in uterine perfusion, and a transient increase in fetal brain blood flow. Maternal glutamine supplementation was able to mitigate alcohol-induced maternal hypercapnea, fetal acidemia and the transient increase in fetal brain blood flow. Thus, this study provides a strong foundation for ongoing studies on assessing the effect of maternal glutamine supplementation to prevent the developmental damage in the fetal brain due to prenatal alcohol exposure.

4. EFFECTS OF GESTATIONAL ALCOHOL EXPOSURE AND L-GLUTAMINE SUPPLEMENTATION ON OXIDATIVE STRESS AND REDOX STATUS

4.1 Introduction

Alcohol is a neurotoxic teratogen and has the potential to cause damage in many regions of the brain [1, 126-129]. Postmortem reports and neuroimaging studies in children clinically affected by heavy prenatal alcohol exposure have shown smaller head and brain size [5, 194-198]. Human studies have reported structural abnormalities and physiological dysfunction in several brain regions, including the cerebral cortex, basal ganglia, thalamus and hypothalamus, hippocampus, and corpus callosum [194, 196, 197, 199-201], but based on both human and animal studies the cerebellum appears to be particularly vulnerable to alcohol exposure during development [18, 202-211].

Oxidative stress is one of the key mediators of fetal neurodevelopmental injury in response to alcohol exposure [212-215]. Evidence of formation of different biomarkers of oxidative stress due to alcohol exposure have been demonstrated in astrocytes [216], neural crest cells [217, 218], cortical neurons [219], cerebellar granule cells [220], and in cerebellar tissue [221, 222]. It has been suggested that alcohol exposure reduces the endogenous anti-oxidant glutathione [223-225]. Although many antioxidants such as vitamin C and E, silymarin, N-acetylcysteine (NAC), melatonin and lazaroid U-83836E have been studied for the potential to mitigate the developmental damage caused by alcohol-induced oxidative stress, there is a great amount of variability in findings based

on the route and period of alcohol exposure, antioxidant treatment, animal model and region analyzed [226-235].

To our knowledge, no study has been done so far to explore the role of L-glutamine supplementation on alcohol-induced oxidative stress and endogenous anti-oxidant levels. Glutamine is a precursor of the cellular endogenous anti-oxidant, glutathione (γ-glutamyl-cysteinyl-glycine). Glutathione participates in many cellular reactions in which it effectively scavenges free radicals and other reactive oxygen species (ROS) directly and indirectly through enzymatic reactions. In such reactions, reduced glutathione (GSH) is oxidized to form glutathione disulfide (GSSG), which is then reduced to GSH by the NADPH-dependent glutathione reductase [236]. Glutathione is the most abundant low-molecular weight thiol and GSH/GSSG is the major redox couple in animal cells [70, 237]. Glutathione synthesis from glutamate, cysteine and glycine is catalyzed sequentially by two cytosolic enzymes, γ-glutamylcysteine synthetase and glutathione synthetase. In the mammalian brain and other tissues (e.g., kidneys, liver and lymphoid tissue) with a high activity of phosphate-dependent glutaminase, glutamine is a major source of glutamate for intracellular GSH synthesis [238]. Glutamine can act as an important molecule enhancing the levels of glutathione to protect against oxidative stress [119]. Therefore, we hypothesize that third trimester-equivalent repeated maternal alcohol exposure would lead to fetal cerebellar oxidative stress and maternal L-glutamine supplementation would mitigate fetal cerebellar oxidative stress and improve endogenous antioxidant levels.

4.2. Materials and methods

4.2.1. Animals

As explained earlier, in the section 2.2.1.

4.2.2. Treatment groups

As explained earlier, in the section 2.2.2.

The number of animals in each group was 6, 6, 6 and 7 for the saline control, alcohol, glutamine and alcohol+glutamine groups, respectively.

4.2.3. Dosing paradigm

The dosing paradigm used in this study is depicted in Figure 1A. Animals received treatment from GD 109 to 132. Alcohol or saline infusions were given intravenously (IV) through a jugular vein catheter over 1 hour from GD 109 to 132, 3 consecutive days per week to mimic a weekend binge drinking pattern. This period of gestation in sheep overlaps with the human third trimester-equivalent brain growth spurt [35, 36]. On gestational day 109, an intravenous catheter (16 gauge, 3.00 in Extended Use Catheter, Jorgensen, Loveland, CO) was placed percutaneously into the jugular vein. On the day of infusions, ewes were connected to the infusion pump by 0830 hr and alcohol or saline was infused continuously over 1 hr. Infusion solutions were delivered intravenously by peristaltic pump (VetFlo® 7701B IV Vet Infusion Pump, Grady Medical, Temecula, CA). The first four doses of alcohol were 1.75, 2, 2.25 and 2.25 g/kg respectively and thereafter were 2.5 g/kg (Figure 1A). The saline control and glutamine groups received a dose of 0.9% saline that was isovolumetric to the alcohol groups. L-

Glutamine powder from Sigma Aldrich (Cat #5792) was completely dissolved in sterile water at a concentration of 4.5% w/v and passed through a 0.2 μm bacteriostatic filter under aseptic conditions. The solution was kept at room temperature and prepared no sooner than 1 to 2 h prior to administration. A 100 mg/kg dose of GLN was administered IV as a 4.5% w/v aqueous bolus three times a day on 3 consecutive days per week. On GD 132±1 baseline maternal blood was collected (0 min) and after that animals received either saline or alcohol infusion as described earlier. Animals from glutamine and alcohol+glutamine group received single bolus of glutamine (100 mg/kg) just before the start of final infusion. Maternal blood alcohol concentration at the end of infusion (60 min) on GD 132 was estimated using an enzymatic assay kit (Quantichrom® ethanol assay kit; BioAssay Systems, Hayward, CA). At the end of 60 min, the ewes were euthanized using an IV injection of sodium pentobarbitone (75 mg/kg). The uterus was removed from the ewe and the fetus was removed. Fetal cerebellar samples were snap-frozen in liquid nitrogen and then stored at -80°C.

4.2.4. Measurement of glutathione (GSH), glutathione disulphide (GSSG), cysteine and cystine

Reduced (GSH) and oxidized (GSSG) glutathione levels were measured in maternal blood cells, maternal plasma and fetal cerebellar lysates. Samples were pretreated to entrap glutathione and cysteine using iodoacetic acid, sodium borate perchloric acid. Extracts were neutralized using 2M potassium carbonate solution. Supernatant was derivatized to S-carboxymethyl glutathione using 25 mM iodoacetic acid in the presence of 2-mercaptatethanol (for the detection of total glutathione and

cysteine) or 40 mM sodium borate (for the detection of reduced glutathione and cysteine). Samples were run separately on HPLC system for the detection of reduced glutathione and free cysteine, and for detection of total glutathione and total cysteine using a Supelco C_{18} guard column (4.6 mm x 5 cm, 20-40 μm; Supelco® Cat # 59644, Sigma-Aldrich, St. Louis, MO) and a Supelco C_{18} column (4.6 mm x 15 cm, 3 μm; Supelco® Cat # 58985, Sigma-Aldrich, St. Louis, MO). Model 717 plus WISP Autosampler was programmed to mix 25 μl of derivatized sample (or standard) with 25 μl of the o-phthalaldehyde (OPA) reagent for 1 min and then inject the derivatized solution into the HPLC column without any delay. Total run time of 16 min was set at the flow rate of 1.1 ml/min. Initial solvent conditions were 97% A, 3% B run for 1 min. A gradient to 86% A, 14% B was ran from 1.1 to 6.5 min. From 6.6 to 9 min, the conditions were maintained at 0% A, 100% B and returned to 97% A, 3% B from 9.1 to 16 min. Mobile phase A was 0.1 M sodium acetate and mobile phase B was HPLC-grade methanol (Fisher Scientific®, Cat # A452-4, Pittsburgh, PA). 2475 Multi wavelength Fluorescence Detector (Waters®, Milford, MA) was set at 220 nm excitation between 0-6 min and 12-16 min, and at 340 nm excitation between 6-12 min. The setting of excitation at 220 nm before 6 min and after 12 min was designed to suppress fluorescence due to other amino acids that react with OPA. Emission was set at 450 nm between 0 to 16 min and gain of the detection was set at 10. Reduced and total glutathione and cysteine were quantified on the basis of authentic standards (GSH, GSSG, cysteine and cystine; Sigma-Aldrich, St. Louis, MO) using the Millennium-32 software and workstation (Waters®, Milford, MA).

4.2.5. Malondialdehyde assay

Approximately 200 mg of fetal cerebellum was homogenized in phosphate buffered saline in the presence of butylated hydroxytoluene (BHT). Malondialdehyde (MDA) as a biomarker of lipid peroxidation was quantified using a spectrophotometric MDA-586 assay kit (BIOXYTECH® MDA-586™, Oxis International Inc., Foster City, CA). Absorbance was detected at 586 nm.

4.2.6. Immunoblotting

Fetal cerebellum was homogenized in an ice cold homogenization buffer containing 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 10 mM Tris, 140 mM NaCl, 10 mM EDTA and 10 µL of Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Rockford, IL). The protein content of lysates was determined using Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL). Each cerebellar homogenate was mixed with 4x NuPage® LDS sample loading buffer (Invitrogen™) containing 10% β-mercaptoethanol and heated in boiling water for 5 min. After the solution was cooled on ice for 2 min, it was used for Western blot analysis. Aliquots of samples with an equal amount of protein were separated by size on 4-20% polyacrylamide gels (150 V, 42 min; Mini Protean II, Bio-Rad Laboratories, Inc., Hercules, CA) alongside Rainbow molecular weight markers (Bio-Rad Laboratories, Inc., Hercules, CA) before transfer to Immobilon P membranes (100 V, 1 h). The Immobilon P membranes were probed using the enhanced chemiluminescence reagent detection system, as described by Amersham Pharmacia Biotech (Arlington Heights, IL), and exposed to Hyperfilm (Amersham Pharmacia Biotech). Antibodies to β-actin were

obtained from Cell Signaling Technologies Inc., superoxide dismutase 1 (SOD) (Abcam Inc.), glutathione s transferase (GST) (Pierce Inc.), S-nitroso-glutathione reductase (GSNOR) (Lifespan Inc.) were also utilized for analysis. All protein measurements were normalized to β-actin.

4.2.6. Statistical analysis

One-way ANOVA was performed for the analysis of all the parameters among groups. Further pairwise comparisons were performed when appropriate using Fisher's protected least significant difference. Level of significance was established at $P<0.05$ and $0.05<P<0.1$ were considered trends.

4.3 Results

4.3.1 BAC

Maternal BACs at the end of final alcohol infusion (60 min; the time point when BACs are known to peak) on GD 132, were 286.78±26.40 mg⁄dl and 297.48±14.53 mg⁄dl in the alcohol and alcohol+glutamine groups, respectively. The differences between the alcohol and alcohol+glutamine groups BAC were not statistically significant.

4.3.2. Maternal blood glutathione and cysteine

Levels of reduced glutathione (GSH) (Figure 8, Panel A-C), oxidized glutathione (GSSG) (Figure 8, Panel D-F) and total glutathione (Figure 9, Panel D-F) in the maternal blood cells were neither altered at the baseline (0 min) nor at the end of infusion (60

min) among groups. The ratio of oxidized to reduced glutathione (GSSG/GSH) in maternal blood cells at baseline was significantly decreased in the alcohol group compared to the saline control group (P=0.014) and alcohol+glutamine group (P=0.039) (Figure 9, Panel A). The ratio of oxidized to reduced glutathione (GSSG/GSH) in maternal blood cells at the end of infusion did not differ significantly among groups (Figure 9, Panel B). Percent change in the GSSG/GSH ratio in the maternal blood cells from 0 to 60 min was significantly increased in alcohol group compared to the saline control (P=0.005), glutamine (P=0.049) and alcohol+glutamine (P=0.029) groups (Figure 9, Panel C). These results indicate that relative levels of oxidized glutathione (GSSG) were increased and reduced glutathione (GSH) were decreased in maternal blood cells after acute alcohol exposure on GD 132, which was followed by the repeated alcohol exposure during the third trimester equivalent period, and maternal glutamine supplementation mitigated this effect.

Levels of GSH, GSSG, ratio of GSSG/GSH and total glutathione in maternal plasma did not differ significantly among groups at baseline (0 min) or at the end of infusion (60 min) on GD 132 (Table 8).

Levels of free cysteine, oxidized cysteine (cystine), ratio of cystine/cysteine and total cysteine in the maternal blood cells (Table 9) and maternal plasma (Table 10) did not differ significantly among groups at the baseline (0 min) or at the end of infusion (60 min) on GD 132.

4.3.3. Fetal cerebellar malondialdehyde (MDA)

Levels of fetal cerebellar MDA were significantly increased in the alcohol group compared to the saline control (P=0.033) and glutamine (P=0.015) groups (Figure 10). Fetal cerebellar MDA levels showed an increasing trend in the alcohol+glutamine group compared to the saline control group (P=0.098) and it was significantly increased compared to the glutamine group (P=0.047). These results indicate that maternal alcohol exposure significantly increased the biomarker of lipid peroxidation in the fetal cerebellum.

4.3.4. Fetal cerebellar glutathione and cysteine

Levels of reduced glutathione (GSH) did not differ between alcohol and saline control groups but were significantly elevated in the alcohol+glutamine group compared to the saline control (P<0.001), alcohol (P=0.005) and glutamine (P<0.001) groups (Figure 11, Panel A). Levels of oxidized glutathione (GSSG) and the ratio of GSSG/GSH were not altered among groups (Figure 11, Panel B and C). Levels of total glutathione were significantly increased in the alcohol+glutamine group compared to the saline control (P<0.001), alcohol (P=0.027) and glutamine (P<0.001) groups. Levels of total glutathione in the alcohol group showed an increasing trend compared to the saline control (P=0.072) and glutamine (P=0.057) groups. No significant difference was observed between the saline control and glutamine groups. These results indicate that repeated maternal alcohol exposure during the third trimester-equivalent period did not alter fetal cerebellar glutathione levels significantly but maternal glutamine supplementation in alcohol exposed fetuses improved fetal cerebellar glutathione levels.

Levels of fetal cerebellar cysteine did not differ significantly among groups (Figure 12, Panel A). Levels of fetal cerebellar cystine (dimer of cysteine) were significantly decreased in the alcohol group compared to the saline control (P=0.046) and glutamine (P=0.012) groups (Figure 12, Panel B). Levels of fetal cerebellar cystine were significantly decreased in the alcohol+glutamine group compared to the glutamine group (P=0.025) (Figure 12, Panel B). The ratio of cystine/cysteine and total cysteine did not differ significantly among groups (Figure 12, Panel C and D).

4.3.5. Fetal cerebellar superoxide dismutase (SOD), Glutathione S-transferase (GST), S-Nitrosoglutathione Reductase (GSNOR)

Levels of fetal cerebellar SOD, GST and GSNOR on GD 132 did not differ among groups (Figure 13).

4.4 Discussion

Five crucial findings can be gathered from this study; first, acute maternal alcohol exposure on GD 132, which was followed by the repeated alcohol exposure during the third trimester-equivalent period, results in a relative increase in oxidized glutathione and a decrease in reduced glutathione in maternal blood; hence leading to a decrease in overall circulating endogenous antioxidant status. Second, maternal glutamine supplementation prevented the alcohol-induced relative increase in oxidized glutathione and decrease in reduced glutathione in maternal blood; hence improving overall endogenous antioxidant status in maternal blood. Third, maternal alcohol exposure leads to fetal cerebellar oxidative stress and maternal glutamine

supplementation partially mitigated this effect. Fourth, maternal glutamine supplementation in alcohol exposed fetuses improved the cerebellar antioxidant status which was evident by an increase in levels of glutathione. Fifth, prenatal alcohol exposure reduces the levels of fetal cerebellar cystine.

4.4.1. Effect of alcohol on biomarkers of oxidative stress

Two step metabolism of alcohol, mediated by the enzymes alcohol dehydrogenase and aldehyde dehydrogenase leads to the generation of reactive oxygen species (ROS) in various tissues [239]. Metabolism of alcohol via cytochrome P450 2E1 also leads to an increase in hydroxyl radical ($^{\bullet}OH$), hence leading to lipid peroxidation [240, 241]. Our finding demonstrates that maternal alcohol exposure leads to fetal cerebellar oxidative stress, which was manifested by an increase in the lipid peroxidation biomarker, MDA. Petkov and colleagues showed that alcohol exposure (9 g/kg) throughout the gestation period and/or during the lactation period leads to an increase in lipid peroxidation biomarkers in the cerebellum as well as other fetal brain regions such as the hippocampus, striatum, hypothalamus and cerebral cortex of 12 week old rats [242]. Another study in the rat model reported that alcohol exposure via liquid diets containing 2%, 4.5%, 6.5%, or 9.25% of alcohol from GD 6 to birth induced increase in the lipid peroxidation biomarker, 4-hydroxy-2,3-nonenal (4-HNE) in fetal cerebellar granule neurons [243]. On the contrary, Kane and colleagues reported that alcohol exposure (6 g/kg) on PND 4 or 14 in neonatal rats does not increase biomarkers of oxidative stress in isolated cerebellar granule neurons [244]. Even short term binge maternal alcohol exposure (4 g/kg, 25% v/v) on GD 17-18 increased the levels of a

number of lipid peroxidation biomarkers such as MDA, conjugated dienes and mitochondrial 4-HNE in the fetal brain [225, 245]. Dong and colleagues reported that acute alcohol exposure (2.9 g/kg) on GD 9 increased the generation of ROS in mouse embryos [246]. Acute alcohol vapor inhalation (peak BAC= 292.5±6.5 mg/dl) during the human third trimester-equivalent of brain growth spurt in the rat on post natal day (PND) 7 resulted in an increase in generation of ROS in the cerebral cortex [247]. Alcohol exposure (2.5 g/kg) on PND 7 increased the levels of thiobarbituric acid reactive substances (TBARS) (biomarker of lipid peroxidation) in the mouse brain on PND 8 [248]. Alcohol exposure at a dose of 6 g/kg on PND 4 and 5 increased the levels of cerebellar TBARS in rat pups [249]. Similarly, Smith and colleagues showed that acute alcohol exposure at a dose of 6 g/kg to rat pups between PND 4 and PND 9 leads to an increase in cerebellar MDA; however no incidence of alcohol-induced lipid peroxidation was observed in the hippocampus or cortex [250]. Acute alcohol vapor inhalation (BAC= 287.0±10.16 mg/dl) on PND 4 was shown to increase ROS production in the cerebellum of rat pups [251]. Collectively these reports strongly support our finding that there is an involvement of oxidative stress in alcohol-induced developmental cerebellar damage.

4.4.2. Effect of alcohol on endogenous antioxidants

Endogenous antioxidant defense mechanisms can be classified under two categories: non-enzymatic and enzymatic [239]. Thiol (-SH) containing molecules such as glutathione and cysteine belong under the non-enzymatic endogenous antioxidant regimen, whereas enzymes such as superoxide dismutase (SOD) and glutathione-s-

transferase, which either inactivate ROS or enhance the efficacy of non-enzymatic endogenous antioxidants, fall under the enzymatic category. We report that acute maternal alcohol exposure, which was followed by repeated alcohol exposure during the third trimester-equivalent period, increased the ratio of oxidized glutathione (GSSG) to reduced glutathione (GSH) in maternal blood cells. However, we did not observe any direct effect of alcohol exposure on fetal cerebellar glutathione levels. Reyes and colleagues reported that maternal alcohol exposure (35% alcohol derived calories) throughout 21 days of gestation in the rat model leads to a decrease in glutathione levels in fetal brain [223]. Dembele and colleagues reported that maternal alcohol exposure at a dose of 2 g/kg throughout 21 days of gestation in the rat model leads to a decrease in hypothalamic glutathione levels on PND 7 and PND 90 [252]. Maternal binge alcohol exposure (4 g/kg, 25% v/v) in pregnant rats on GD 17-18 decreased the levels glutathione in the fetal brain by 19% [225]. On the contrary, Smith and colleagues showed that alcohol exposure at a dose of 6 g/kg to rat pups between PND 4 and PND 9 leads to an increase in cerebellar glutathione levels [250]. A study in a pregnant guinea pig model (approximate gestation period 68 days) showed that chronic oral administration of alcohol at a dose of 4 g/kg throughout gestation decreased fetal and neonatal hippocampal as well as brain weight, but surprisingly no change in fetal mitochondrial glutathione or fetal and neonatal cytosolic glutathione concentration was observed; however, neonatal mitochondrial glutathione concentration was decreased [224].

We observed that repeated maternal alcohol exposure during the third trimester-equivalent period decreased the levels of fetal cerebellar cystine but levels of cysteine were unaltered. Sufficient bioactive concentration of thiols such as cysteine, cystine and other sulfhydral (-SH) containing compounds is very crucial for defining the total antioxidant status of tissue. Along with other two amino acids, glutamate and glycine, cysteine is a part of tri-peptide, glutathione [70, 72]. Efforts have been made to investigate the ability of the cysteine precursor, N-acetyl-cysteine (NAC) to attenuate alcohol-induced developmental damage. A study done using NAC as an intervention agent against a single alcohol exposure (6 g/kg) on PND 4 in rat pups reported no significant effect on alcohol-induced Purkinje cell loss [231]. On the contrary, Parnell and co-workers have reported a significant effect of NAC against alcohol-induced fetal ocular abnormalities in the mouse model [253]. In another study, a significant effect of NAC was observed to normalize alcohol-induced MDA and glutathione contents in fetal rat peripheral nerves [254]. However, none of these reports measured actual concentration of cysteine or cystine in fetal tissues. Therefore, our finding is the first to report the effect of maternal alcohol exposure on fetal cerebellar cysteine and cystine.

Although, many investigators have evaluated the role of the enzymatic antioxidant system against alcohol-induced developmental damage, there are several differences among these findings. In a recent study, Ramezani and colleagues observed no changes in rat cerebellar superoxide dismutase (SOD) activity 90 min after second alcohol exposure (6 g/kg) on PND 5 which was followed by the first alcohol exposure on PND 4 [249]. Dembele and colleagues reported that maternal alcohol exposure (2 g/kg)

throughout 21 days of gestation leads to an increase in hypothalamic Mn SOD expression in adult rats (PND 90) but not in neonatal rats (PND 7), whereas expression of hypothalamic Cu/Zn SOD was unaltered in neonatal as well as adult rats exposed to alcohol *in-utero* [252]. Extensive work by Heaton and colleagues demonstrated that alcohol exposure during the early postnatal period in the rat model exhibits temporal and regional differences in expression of SOD in the brain [247, 255, 256]. Dong and colleagues reported that acute alcohol exposure (2.9 g/kg) on GD 8 increased the mRNA expression of SOD and GST in mouse embryos [257]; whereas, Devi and colleagues reported that incubation of cultured fetal rat hepatocytes in media containing 150-200 mg/dl of alcohol for 6 h did not alter the levels of SOD and GST [258]. In our current study, we did not observe any alterations in fetal cerebellar SOD, GST and GSNOR activity at the end of final alcohol exposure on GD 132. However, it should be noted that defense mechanisms of the developing brain against oxidative stress are limited, and GSH and GST levels in fetal rat brain on GD 19 were only 51% and 11% of the adult values, respectively [259]. All together these findings imply that the dynamics of the endogenous antioxidant system in response to alcohol exposure are attributed to differences in the timing and degree of alcohol exposure, species differences and regional differences. It should be noted that most of studies discussed here did not quantify reduced and oxidized glutathione separately and minimal attention was given to the thiol biochemistry.

4.4.3. Effect of glutamine on glutathione levels

In the current study, we observed that maternal glutamine supplementation corrected the alcohol-induced relative decrease in reduced glutathione (GSH) and increase in oxidized glutathione (GSSG) in maternal blood. We also observed an increase in the levels of fetal cerebellar reduced glutathione (GSH) and total glutathione levels in the glutamine supplemented alcohol group. A study in the rat model demonstrated that glutamine supplementation attenuated acetaminophen-originated oxidative toxicity-induced decrease in hepatic glutathione [260]. Oral glutamine supplementation has been shown to mitigate anti-cancer drug, cyclophosphamide-induced oxidative stress in the rat model of hemorrhagic cystitis [261]. Manhart and colleagues showed that glutamine administration in mice prevented lipopolysaccharide-stimulated lymphocyte atrophy in Peyer's patches by increasing glutathione levels [262]. It was postulated that supplementation of glutamine in the clinical diet can be used to maintain high levels of glutathione and to avoid oxidative stress [263] and a clinical study done in patients from a surgical intensive care unit showed that glutamine fortified parental nutrition improved the levels of plasma glutathione levels compared to the normal non-glutamine fortified parental nutrition [264]. Collectively these preclinical and clinical reports support our findings that maternal glutamine supplementation attenuates alcohol-induced alterations in glutathione levels.

In summary, results from this chapter suggest that alcohol exposure leads to significant alterations in the redox status of the mother and fetus, and glutamine supplementation has potential to improve the endogenous antioxidant capacity.

5. DIFFERENTIAL ALTERATION IN FETAL CEREBELLAR AND SKELETAL MUSCLE MTOR SIGNALING PATHWAY IN RESPONSE TO MATERNAL ALCOHOL EXPOSURE AND GLUTAMINE SUPPLEMENTATION

5.1. Introduction

The evolutionarily conserved mammalian target of rapamycin (mTOR) was recognized after the discovery of TOR genes in the *Saccharomyces cerevisiae* during the screening for resistance to the drug rapamycin [265-269]. The signaling pathway through mTOR contributes to cell size, growth and differentiation by governing transcription, ubiquitin–dependent proteolysis and microtubule stability [270-272]. Studies have shown that phosphorylation of mTOR regulates protein synthesis through the phosphorylation and activation of S6 kinase (S6K1) [273]; S6K1 is involved with phosphorylation of the 40S ribosomal S6 protein, which governs protein synthesis rate by mediating protein translational initiation and elongation [274, 275]. Phosphorylation of mTOR also regulates the phosphorylation and inactivation of the repressor of mRNA translation, eukaryotic initiation factor 4E-bindin protein (4E-BP1) [276].

Alcohol exposure alters amino acid homeostasis and it is known that bioavailability of amino acids regulate the mTOR signaling pathway in various tissues and cell lines [118, 277-283], but no research has been done to evaluate the simultaneous effect of maternal alcohol exposure and glutamine supplementation on the mTOR signaling pathway. The mTOR signaling pathway has been associated with maintaining overall cellular growth, synaptic plasticity, dendritic branching and neuronal development [284-287] and we know that prenatal alcohol exposure results in fetal

growth deficits and neuronal insult. Therefore, we speculate that prenatal alcohol exposure and alcohol-induced perturbations in amino acid availability may lead to alterations in the mTOR signaling pathway in the fetal cerebellum and skeletal muscle, and this may be one of the underlying mechanisms of alcohol-induced developmental damage. To our knowledge, no study has been done so far to investigate the role of the mTOR signaling pathway in fetal developmental damage caused by prenatal alcohol exposure using an *in-utero* model. Therefore, we hypothesized that the third trimester-equivalent alcohol exposure would alter the fetal mTOR signaling pathway and maternal glutamine supplementation would ameliorate the effects of alcohol on the mTOR signaling pathway.

5.2. Material and Methods

5.2.1. Animals

As explained earlier, in the section 2.2.1.

5.2.2. Treatment groups

As explained earlier, in the section 2.2.2.

The number of animals in each group was 6, 6, 6 and 7 for the saline control, alcohol, glutamine and alcohol+glutamine groups, respectively.

5.2.3. Dosing paradigm

As explained earlier, in the section 4.2.3.

5.2.4. *Immunoblotting*

Fetal cerebellum was homogenized in an ice cold homogenization buffer containing 1% sodium deoxycholate, 1% NP-40, 0.1% SDS, 10 mM Tris, 140 mM NaCl, 10 mM EDTA and 10 µL of Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, Rockford, IL). The protein content of lysates was determined using Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL). Each cerebellar homogenate was mixed with 4x NuPage® LDS sample loading buffer (Invitrogen™) containing 10% β-mercaptoethanol and heated in boiling water for 5 min. After the solution was cooled on ice for 2 min, it was used for Western blot analysis. Aliquots of samples with an equal amount of protein (50 µg) were loaded onto SDS–polyacrylamide gels. After separation on 4–12% gels, proteins were transferred to a PVDF membrane (0.45 µm) (Thermo Fisher Scientific, Rockford, IL) under 12 V overnight, using the Transblot apparatus (Bio-Rad, Hercules, CA). Membranes were blocked in either 3% BSA (for phosphorylated proteins) or 5% fat-free milk (for total proteins) in Tris-Tween buffered saline (TTBS; 20-mM Tris/150-mM NaCl, pH 7.5, and 0.1% Tween-20) for 2-3 hours. Blocked membranes incubated with the following primary antibodies overnight at 4^{o}C with gentle rocking: mTOR, phosphorylated mTOR (Ser2448), p70/85 S6K1, phosphorylated p70/85 S6K1 (Thr421/Ser424), 4E-BP1, phosphorylated 4E-BP1 (Thr70), Akt, phosphorylated Akt (Thr308) or β-actin. After washing five times with TTBS, the membranes were incubated at room temperature for 1.5 h with Anti-rabbit IgG, HRP-linked secondary antibody. All antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Finally, the membranes

were washed with TTBS, followed by development using Supersignal® Dura Extended Duration Substrate according to the manufacturer's instructions (Thermo Fisher Scientific, Rockford, IL). The signals were detected on ChemiDOC™ XRS system (Bio-Rad, Hercules, CA). The density of bands was quantified using Quantity One® 1-D analysis software. All protein measurements were normalized to β-actin.

5.2.5. Statistical analysis

One-way ANOVA was performed for the analysis of all the parameters among groups. Further pairwise comparisons were performed when appropriate using Fisher's protected least significant difference. Level of significance was established at $P<0.05$ and $0.05<P<0.1$ were considered trends.

5.3. Results

5.3.1. Maternal BAC

The mean ± SEM maternal BACs at the end of ethanol infusion (1 hour; the time point when BACs are known to peak), were 327.97±16.02 mg⁄dl and 316.18±29.10 mg⁄dl in the alcohol and alcohol+glutamine treatment groups, respectively. The difference between the alcohol and alcohol+glutamine treatment groups BAC was not statistically significant.

5.3.2. Fetal cerebellar and skeletal muscle mTOR

Fetal cerebellar phosphorylated mTOR levels did not differ significantly among groups (Figure 14, Panel A). Levels of total mTOR were significantly decreased in the

alcohol (P=0.011) and alcohol+glutamine (P<0.001) group compared to the saline control group. Levels of total mTOR showed decreasing trend in the alcohol (P=0.051) and decreased significantly in the alcohol+glutamine (P=0.004) group compared to the glutamine group (Figure 14, Panel B). Levels of total mTOR did not differ between saline control and glutamine groups and between alcohol and alcohol+glutamine groups. Ratio of phosphorylated mTOR to total mTOR was significantly increased in the alcohol+glutamine group compared to the saline control (P=0.003) and glutamine (P=0.014) groups (Figure 14, Panel C). Ratio of phosphorylated mTOR to total mTOR did not differ between saline control and glutamine groups and between alcohol and alcohol+glutamine groups.

Levels of phosphorylated and total mTOR did not differ significantly among groups in fetal skeletal muscles (Figure 15, Panel A and B). However, ratio of phosphorylated mTOR to total mTOR was significantly decreased in the glutamine (P=0.012) and alcohol+glutamine (P=0.003) groups and exhibited a decreasing trend in the alcohol group (P=0.078) compared to the saline control group (Figure 15, Panel C). Ratio of phosphorylated mTOR to total mTOR did not differ between alcohol and alcohol+glutamine groups.

5.3.3. Fetal cerebellar and skeletal muscle S6 kinase (S6K)

Fetal cerebellar Phosphorylated S6K and total S6K did not differ significantly among groups (Figure 16, Panel A and B). The ratio of phosphorylated S6K to total S6K was significantly increased in the alcohol and alcohol+glutamine groups compared to the saline control (P=0.018 and <0.001, respectively) and glutamine (P=0.008 and <0.001,

respectively) groups (Figure 16, Panel C). Ratio of phosphorylated S6K to total S6K did not differ between saline control and glutamine groups and between alcohol and alcohol+glutamine groups.

Levels of fetal skeletal muscle phosphorylated S6K were significantly decreased in the glutamine (P=0.046) and alcohol+glutamine (P=0.029) groups compared to the alcohol group (Figure 17, Panel A). Levels of phosphorylated S6K showed decreasing trend in the alcohol+glutamine group compared to the alcohol group (P=0.078). Levels of fetal skeletal muscle total S6K did not differ among the groups (Figure 17, Panel B). The ratio of phosphorylated S6K to total S6K was significantly decreased in the glutamine group compared to the saline control (P=0.023) and alcohol (P=0.015) groups (Figure 17, Panel C). Ratio of phosphorylated S6K to total S6K did not differ between saline control and alcohol groups and between alcohol and alcohol+glutamine groups.

5.3.4. Fetal cerebellar and skeletal muscle 4 eukaryotic initiation factor binding protein1 (4E-BP1)

Levels of fetal cerebellar phosphorylated 4E-BP1 were significantly decreased in the alcohol+glutamine group (P=0.009) and showed decreasing trend in the alcohol group (P=0.091) compared to the glutamine group (Figure 18, Panel A). Levels of fetal cerebellar phosphorylated 4E-BP1 showed decreasing trend in the alcohol+glutamine (P=0.086) group compared to the saline control group. Levels of total 4E-BP1 and the ratio of phosphorylated 4E-BP1 to total 4E-BP1 did not differ significantly among groups in fetal cerebellum (Figure 18, Panel B and C).

Levels of phosphorylated 4E-BP1, total 4E-BP1 and the ratio of phosphorylated 4E-BP1 to total 4E-BP1 did not differ significantly among groups in fetal skeletal muscle (Figure 19, Panel A, B and C).

5.4. Discussion

Abundant research over last two decades has shown that mammalian target of rapamycin (mTOR) and its downstream regulators, S6K and 4E-BP are associated with cell survival, growth, neurodevelopment, autophagy, aging and cancer biology. Alterations in the mTOR signaling pathway is involved with neurological disorders such as fragile X syndrome and other autism spectrum disorders which share an overlapping link with fetal alcohol syndrome [288-290]. To our knowledge, no research has been done to understand the role of this novel signaling pathway in the field of FASD and as a potential target for development of effective therapeutic intervention. The aim of our study was to understand the effect of maternal alcohol consumption and glutamine supplementation during the third trimester-equivalent period on fetal cerebellar and skeletal muscle mTOR signaling cascade. Three important findings can be drawn from this study; first, third trimester-equivalent maternal alcohol exposure decreases the overall expression of fetal cerebellar total mTOR protein, and no significant effect of glutamine supplementation was observed. Second, maternal alcohol exposure results in mTOR independent activation of fetal cerebellar S6 kinase. Third, maternal glutamine supplementation leads to the inactivation of fetal skeletal muscle S6 kinase in mTOR dependent manner.

Although, to our knowledge, this is the first *in-utero* study to comprehensively evaluate the effect of prenatal alcohol exposure on the fetal mTOR signaling pathway, two separates studies [291, 292] explored the effect of developmental alcohol exposure on mediators associated with the mTOR signaling pathway in rodent models. A study in postnatal rats found that alcohol exposure (5 g/kg) between postnatal day 6-8 leads to a decrease in phosphorylated Akt and alcohol exposure between either postnatal day 2-4, 6-8 or 13-15 hampers the levels of phosphorylated mitogen associated protein kinase (MAPK) and p70 S6K in the cerebral cortex [291]. Cerebellar neuronal cultures generated from alcohol-exposed rat pups exhibited reduced levels of insulin-stimulated phosphorylated Akt and other mediators suggesting that insulin-stimulated survival mechanisms in the developing cerebellum were hampered by the gestational alcohol exposure [292]. Li and Ren demonstrated that chronic alcohol (4%) exposure for 16 weeks in adult mice reduces the activation of mTOR, p70 S6K and 4E-BP1in Akt independent manner in the cerebral cortex [279]. Vary and colleagues have shown that acute alcohol exposure (BAC~380 mg/dl) in adult rats decreased the phosphorylation of myocardial mTOR, S6K1 and 4E-BP1 [293]. In another study, Li and Ren demonstrated that chronic alcohol (4%) exposure for 16 weeks in adult mice led to a decrease in expression of total Akt, phosphorylated mTOR, and phosphorylated p70 S6K-to-p70 S6K ratio, but Phosphorylated Akt, total mTOR, and phosphorylated p70 S6K were unaffected in cardiomyocytes [280]. These findings support our results that prenatal alcohol exposure leads to alterations in the mTOR signaling pathway in the fetal cerebellum. However, it is important to note that we observed an activation of cerebellar

S6K in mTOR independent manner in the alcohol exposed groups. An interesting and novel study by Neasta and colleagues showed that alcohol administration for 3 weeks in C57BL/6J mouse model lead to an activation of the mTOR signaling cascade, which was manifested by increased levels of phosphorylated S6K and 4E-BP, in the nucleus accumbens of mice brains. They also demonstrated that rapamycin, an inhibitor of mTORC1, decreased the expression of alcohol-induced locomotor sensitization and place preference, as well as excessive alcohol intake, hence demonstrating the association between mTOR signaling pathway and alcohol preference [281]. Acetaldehyde, the major metabolite of alcohol, suppressed the phosphorylation of Akt, mTOR and 4E-BP1, but increased the phosphorylation of p70 S6K in dopaminergic SH-SY5Y human neuroblastoma cells. This acetaldehyde-induced activation of p70 S6K was diminished in the presence of the mTOR inhibitor rapamycin, and was unaffected in the presence of insulin, hence collectively suggesting that alcohol and its metabolites could activate ribosomal S6 kinase independent of Akt or mTOR [294].

Activation of S6K depends on the phosphorylation at different sites, and some of the sites of S6K phosphorylation have been identified as independent of the rapamycin-sensitive pathway [295-297]. S6K phosphorylation is governed by both protein kinase C (PKC)-dependent and –independent mechanisms and PKC independent mechanisms are shown to be dependent on activation of PI3K [298, 299]. However, it is necessary to note that many reports have demonstrated that regulation of S6K in the downstream mTOR signaling pathway is in a mTOR necessary but independent way and input from PI3K is, at least in part, independent of mTOR [300-302]. Nonetheless, it is more

important to note that studies in animal models, cell cultures and in humans have shown that alcohol exposure increases the levels of PKC in brain, neural cell culture and human platelets [303-305]. Therefore, based on the above findings from different studies, we speculate that the activation of S6K in fetal cerebellum after prenatal alcohol exposure might be due to alcohol-induced activation of the PKC pathway.

Recent studies have demonstrated that some branched chain amino acids (BCAA), especially L-leucine play a crucial role in activation of S6K and 4E-BP1 through activation of the mTOR signaling pathway in many cell types [278, 306, 307]. However, there have been bilateral findings about the effect of L-glutamine on the mTOR signaling pathway. Xi and colleagues showed that glutamine incubation increased the phosphorylation of S6K1 and 4E-BP1 independent of mTOR activation in porcine intestinal epithelial cells [277]. In another study, Nakajo and colleagues demonstrated that L-glutamine inhibited the arginine and leucine-induced activation of p70 S6K and phosphorylation of 4E-BP1 in rat intestinal epithelial cells [308]. Krause and colleagues documented that glutamine incubation increased the cell volume and induced an anabolic response which was characterized by the activation of p70 S6K, acetyl-CoA carboxylase and glycogen synthase in the rat hepatocytes [283]. Xia and colleagues showed that glutamine incubation increased the phosphorylation of p70 S6K1 (Thr389) in mTOR dependent but PI3K independent manner in rat neonatal cardiomyocytes [282]. Thus, above findings collectively demonstrate that glutamine has differential effects on the mTOR signaling pathway and activation of S6 kinase could be either mTOR dependent or independent based on cell and tissue types. Recently, it was

shown that leucine dependent mTORC1 activation is executed in a glutamine dependent fashion. Glutamine is imported into the cell through the SLC1A5 transporter in a Na^+ dependent manner, and then this imported glutamine is exported for import of leucine via the antiporter SLC7A5 [solute carrier family 7 (cationic amino acid transporter, y+ system, member 5] and SLC3A2 [solute carrier family 3 (activators of dibasic and neutral amino acid transport) member 2], and then this intracellular leucine activates mTORC1 [309, 310]. However, it is important to understand the compartmental complexity of the pregnant animal model system from a physiological standpoint, as well as the differential metabolism of the glutamine molecule in order to differentiate between direct and indirect effects of glutamine. L-glutamine is capable of undergoing transamination to yield other amino acids. Human and animal studies have indicated that L-glutamine supplementation enhances the synthesis and bioavailability of other amino acids. We speculate that regionally differential effect of alcohol and glutamine on the mTOR signaling cascade could be due to the activation of diverse pathways in the presence and absence of alcohol.

5. SUMMARY, SIGNIFICANCE AND PERSPECTIVE

Alcohol consumption by pregnant women has a wide range of negative developmental effects on the fetus. The prevalence of FASD has been estimated to be approaching 1 per 100 live births in high risk populations with an approximation of 40,000 infants being born each year with FASD [311]. It is estimated that the annual economic cost of FAS alone is more than $4 billion in the United States [311]. The National Institute of Alcohol Abuse and Alcoholism (NIAAA) have stated four main objectives in their strategic plan for fiscal year 2009-2013. They are: improve understanding of FASD prevalence, develop interventions to prevent and mitigate FASD, improve FASD diagnosis and elucidate mechanisms of FASD pathogenesis and develop interventions for the treatment of FASD. In coherence with the fourth objective, this work using the sheep model not only investigates the effect of prenatal alcohol exposure on fetal growth, cardiovascular and neuropathology, but also contributes towards developing interventions for the treatment of FASD.

In the first study, we demonstrated that repeated maternal alcohol exposure during the third trimester-equivalent period alters amino acid homeostasis and leads to fetal intra-uterine growth restriction (IUGR). Maternal glutamine supplementation was able to prevent alcohol-induced alterations in amino acid bioavailability and improved fetal growth. Perturbations during gestation can have detrimental effects on the post natal development of offspring. Nutritional disturbances during the period of development can be associated with an increased risk of early onset of various diseases in the future. Findings from this study not only demonstrate that alcohol-induced

imbalances in amino acids are directly or indirectly responsible for fetal growth restriction but also creates a foundation for designing nutrition based therapeutic interventions to ameliorate alcohol-induced IUGR.

Although the cardiovascular adaptations of offspring during perinatal, neonatal, juvenile, adolescent and adulthood period are determined by a number of factors, altered fetal programming during gestation may influence development of cardiovascular disorders in the future. Since it has been very recent that researchers in the field of FASD have focused their attention on fetal cardiovascular complications, effects of gestational alcohol exposure on the long-term cardiovascular health of offspring are unknown. Results from this study show that repeated prenatal alcohol exposure in sheep during the human third trimester-equivalent period of brain development produces maternal and fetal acidemia, hypercapnea and maternal but not fetal hypoxemia, elevation in fetal MAP, decrease in uterine perfusion, and alterations in fetal brain blood flow. Maternal glutamine supplementation was able to mitigate alcohol-induced maternal hypercapnea, fetal acidemia and the concurrent transient increase in fetal brain blood flow, hence limiting alcohol delivery to fetal brain. Considering the devastating role of alcohol and the fact that the incidence of maternal drinking during pregnancy has not gone down, with an estimated incidence of 2-5% of the U.S. population [8, 12, 127, 312-315], it is very important to conduct multi-institutional preclinical and clinical investigations to understand the long term effect of prenatal alcohol exposure on cardiovascular adaptations in offspring.

Oxidative stress is one of the mechanisms by which alcohol exhibits its teratogenic effect. Extensive research done using *in-vivo* and *in-vitro* models highlight that alcohol-induced oxidative stress exhibits multifaceted alterations at the cellular level. Our study using a third trimester-equivalent pregnant sheep model shows that maternal alcohol exposure causes significant alterations in the redox status of the mother as well as fetus and maternal glutamine supplementation improved the levels of fetal cerebellar glutathione.

Based on findings from the last study it can be concluded that alcohol and glutamine result in differential alterations in the mTOR signaling cascade. Considering the regional intricacies of the mTOR signaling pathway and its involvement with axonal regeneration, dendritic arborization, synaptic plasticity, cellular growth and autophagy, it is highly likely that there is an association between mTOR signaling pathway molecules and alcohol. However, S6K and 4E-BP1 are not the only downstream targets of mTOR, nor the straight forward PI3K-Akt-TSC1/2-Rheb pathway the only upstream regulator of mTOR. More must be deciphered about the complexity of the mTOR signaling cascade to understand the effect of alcohol on this pathway. In coming years, powerful high-throughput genomic and proteomic tools will provide more understanding of the complexity of this signaling pathway to design an effective therapeutic treatment for not only FASD, but also extend our knowledge about other developmental disorders.

Considering the multi-faceted role of the glutamine molecule, further investigations are warranted to broaden our understanding about the role of glutamine during development. Findings from these studies form a strong foundation for future

studies to investigate the effect of glutamine against alcohol-induced fetal growth retardation, cardiovascular complications and neuropathology during fetal development.

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APPENDIX A

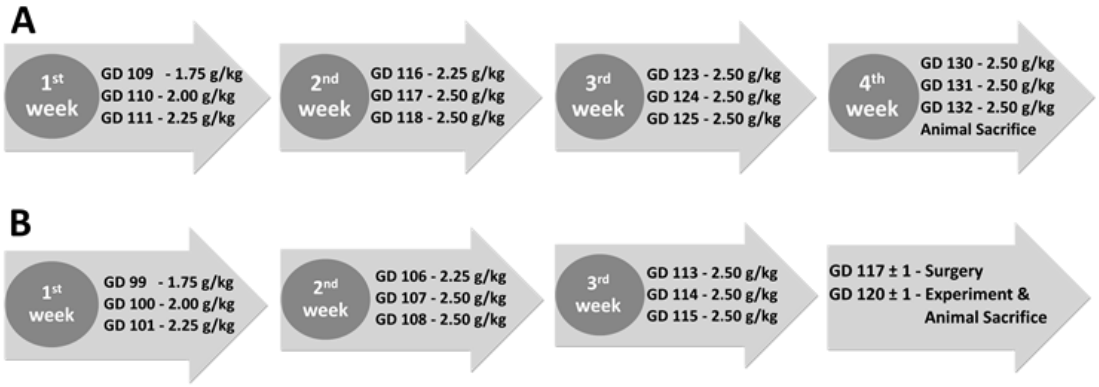

Figure 1: Treatment paradigm
Description of weekend binge alcohol drinking paradigm followed from gestation day (GD) 109 to 132 for experiment 1 (Panel A) and from GD 99 to 115 for experiment 2 (Panel B) using the third trimester-equivalent sheep model. Maternal glutamine supplementation was done in the same pattern with a dose of 100 mg/kg three times per day. Average gestation period of sheep is 147-150 days.

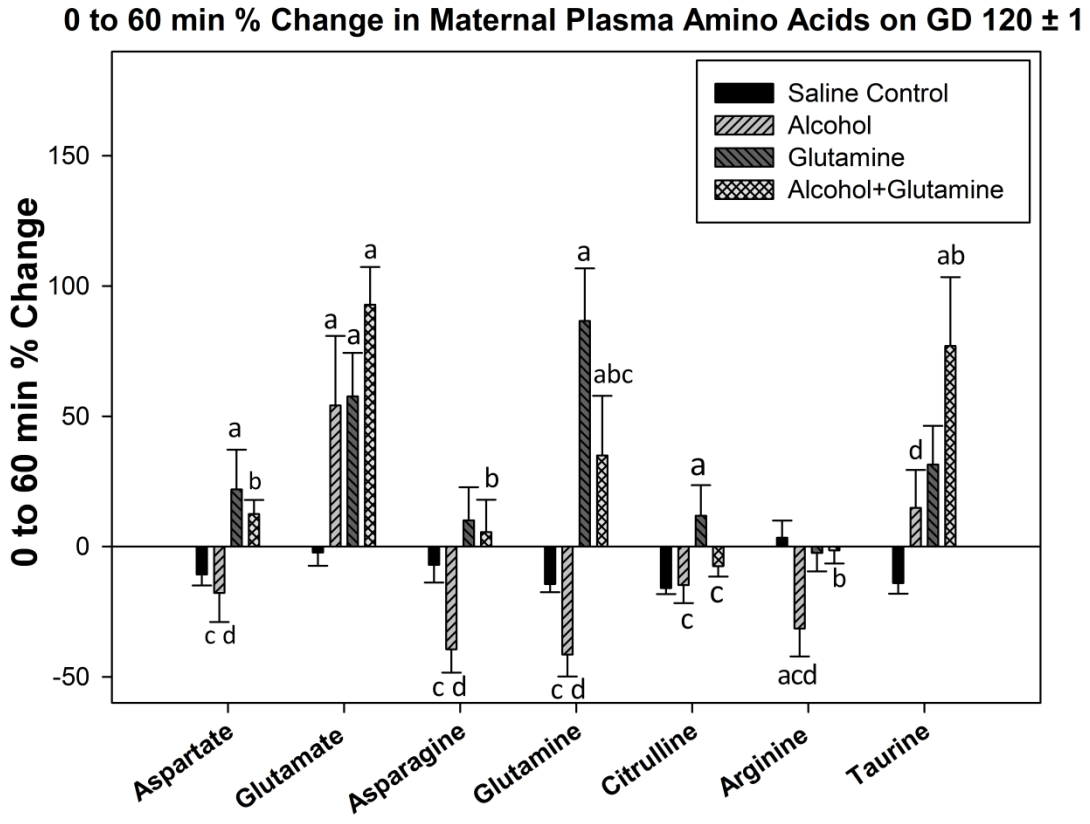

Figure 2: 0 to 60 min percent change in maternal plasma amino acids
Percent change in maternal arterial plasma amino acid concentrations at the end of infusion (60 min) from the baseline (0 min) on GD 120±1. **a**, **b**, **c** and **d** indicate statistically significant differences ($P<0.05$) compared to the saline control, alcohol, glutamine and alcohol+glutamine groups, respectively.

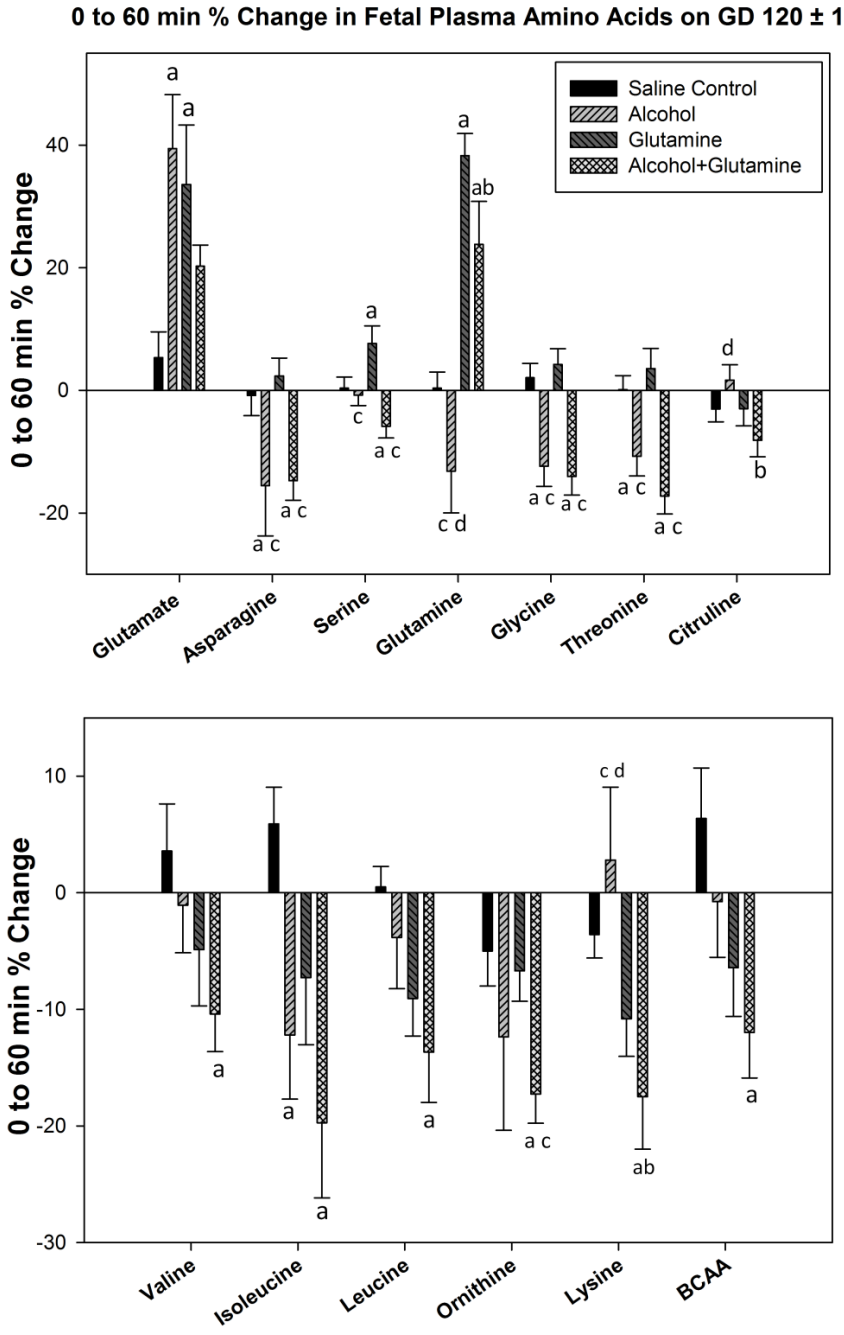

Figure 3: 0 to 60 min percent change in fetal plasma amino acids

Percent change in the fetal arterial plasma amino acid concentrations at the end of infusion (60 min) from the baseline (0 min) on GD 120±1. **a**, **b**, **c** and **d** indicate statistically significant differences ($P<0.05$) compared to the saline control, alcohol, glutamine and alcohol+glutamine groups, respectively.

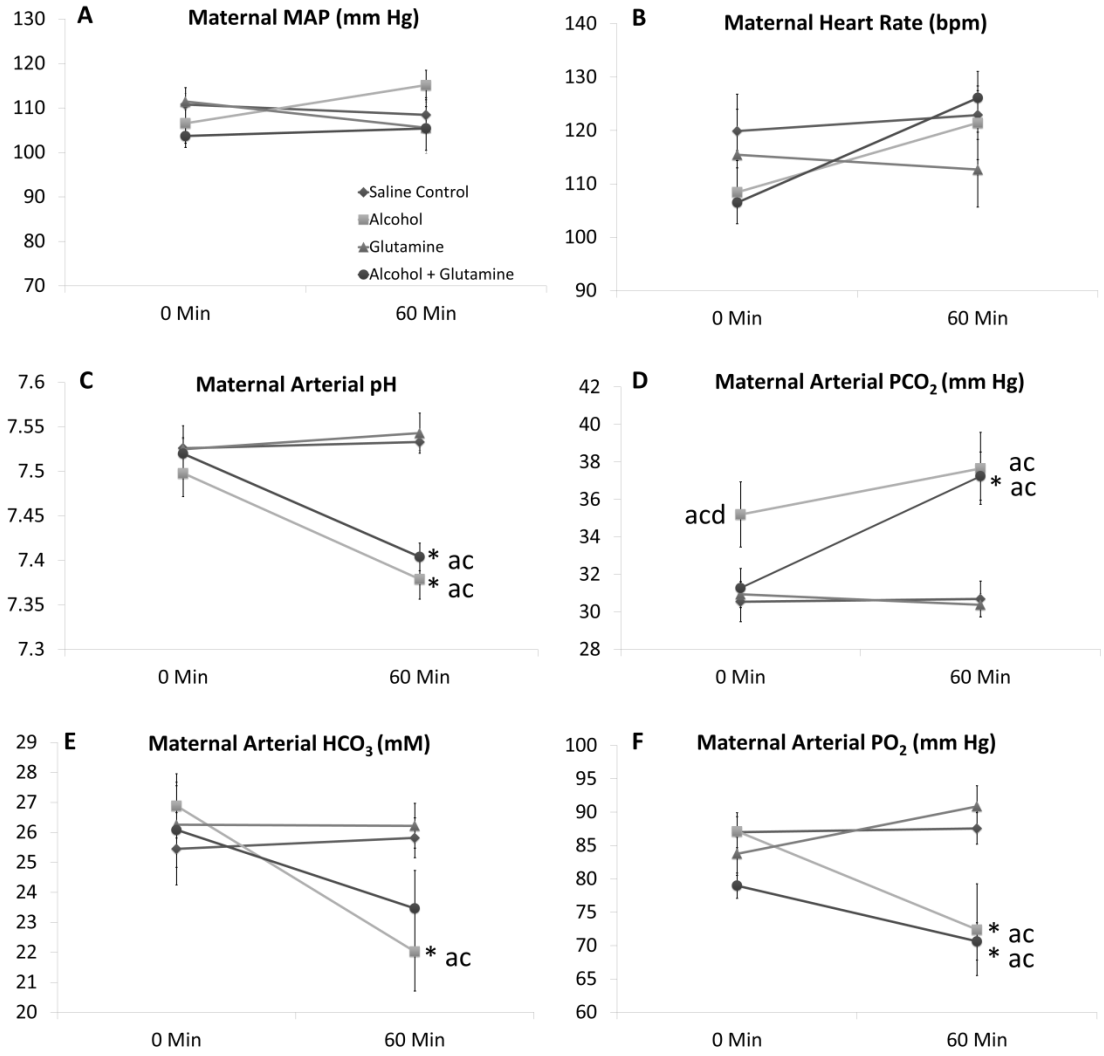

Figure 4: Maternal systemic hemodynamic parameters on GD 120±1

No changes were observed for maternal MAP (Panel A) and HR (Panel B) among groups at the baseline (0 min) or at the end of infusion (60 min). Maternal arterial pH was decreased at the end of 60 min in the alcohol and alcohol+glutamine groups (Panel C). A higher level of maternal arterial PCO_2 was observed at the baseline (0 min) in the alcohol group and maternal arterial PCO_2 was increased at the end of 60 min in the alcohol and alcohol+glutamine groups (Panel D). Concentration of maternal arterial bicarbonate (HCO_3) was decreased at the end of 60 min in the alcohol group (Panel E). Maternal arterial PO_2 was decreased at the end of 60 min in the alcohol and alcohol+glutamine groups (Panel F). **a**, **b**, **c** and **d** indicate statistically significant differences ($P<0.05$) compared to the saline control, alcohol, glutamine and alcohol+glutamine groups, respectively. ***** indicate statistically significant differences ($P<0.05$) in the alcohol and/or alcohol+glutamine group compared to their respective baseline.

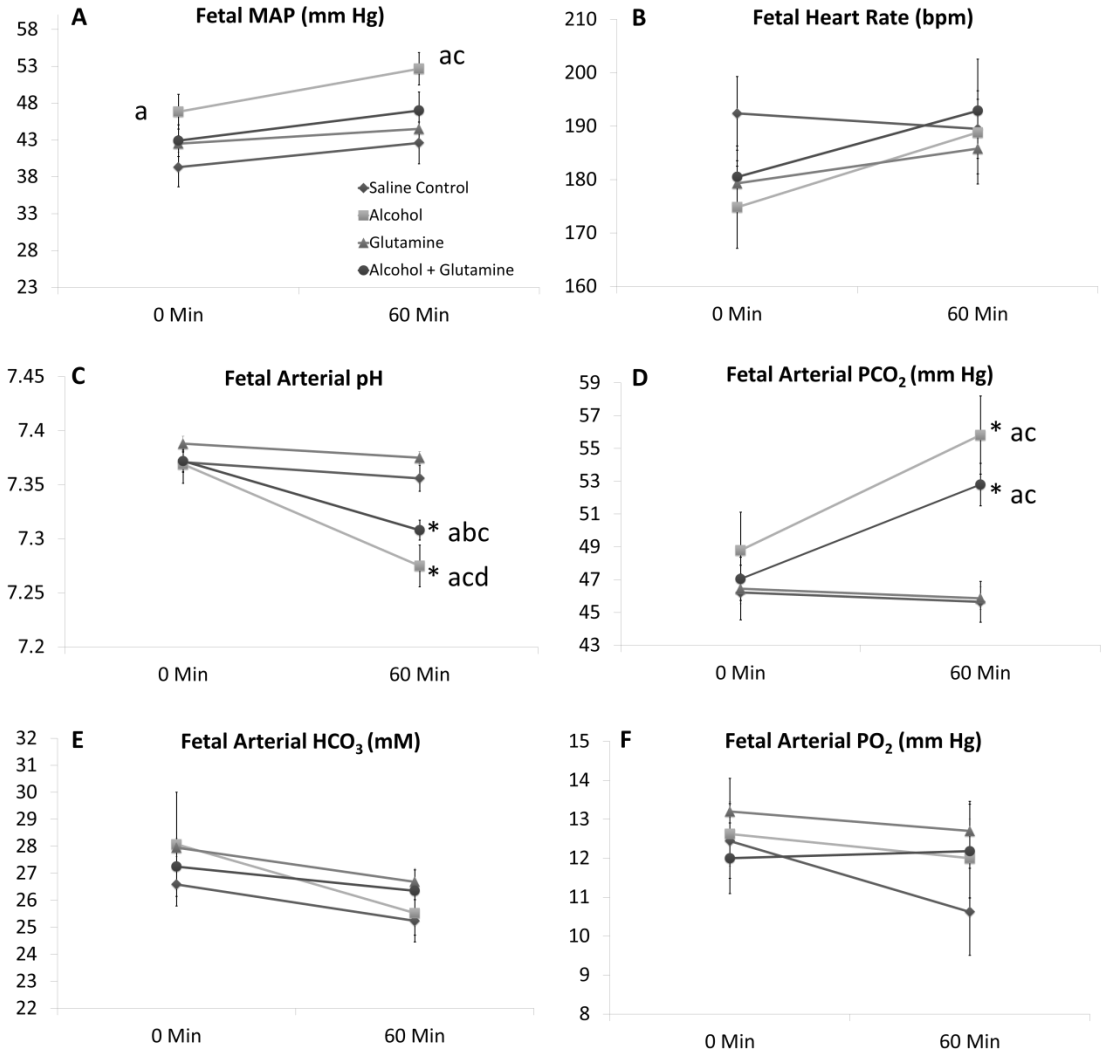

Figure 5: Fetal hemodynamic parameters on GD 120±1

Fetal MAP was elevated at the baseline (0 min) and at the end of infusion (60 min) in the alcohol group (Panel A). Fetal HR did not vary significantly among groups at the baseline and at the end of infusion (Panel B). Fetal arterial pH was decreased at the end of 60 min in the alcohol and alcohol+glutamine groups and this decrease in the alcohol group was significantly greater than the alcohol+glutamine group (Panel C). Fetal arterial PCO_2 was increased at the end of 60 min in the alcohol and alcohol+glutamine groups (Panel D). Fetal arterial bicarbonate (HCO_3) (Panel E) and PO_2 (Panel F) did not differ among groups at 0 or 60 min. **a**, **b**, **c** and **d** indicate statistically significant differences ($P<0.05$) compared to the saline control, alcohol, glutamine and alcohol+glutamine groups, respectively. ***** indicate statistically significant differences ($P<0.05$) in the alcohol and/or alcohol+glutamine group compared to their respective baseline.

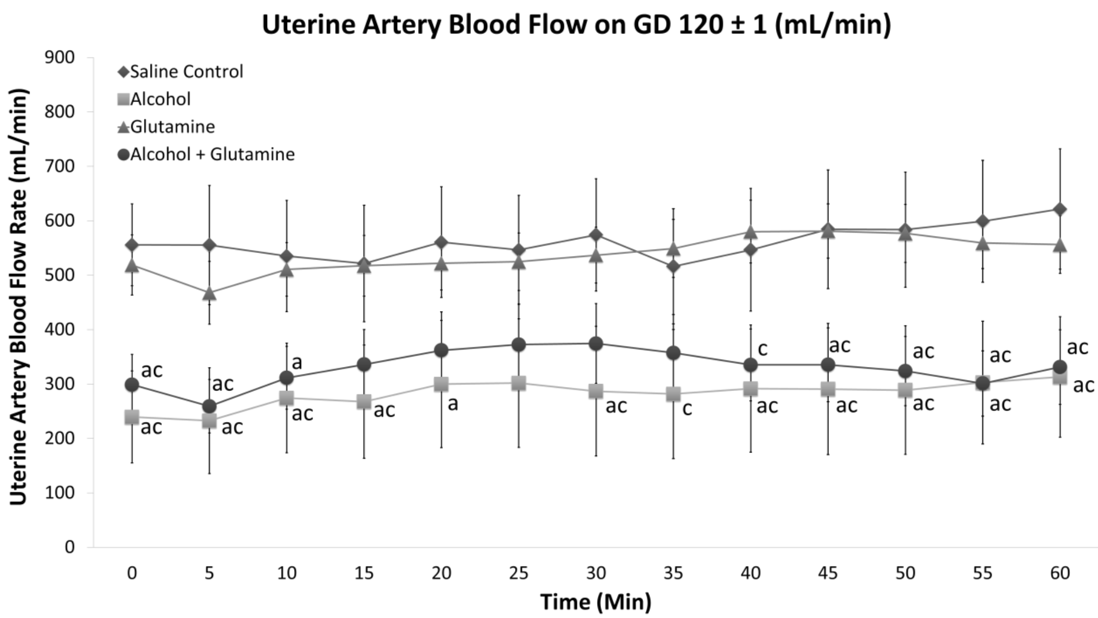

Figure 6: Uterine artery blood flow rate

Uterine artery blood flow (mL/min) was significantly decreased in the alcohol and alcohol+glutamine groups at the baseline (0 min) and during the period of entire infusion (0-60 mins) on GD 120±1. **a** and **c** indicate statistically significant differences (P<0.05) compared to the saline control and glutamine groups, respectively.

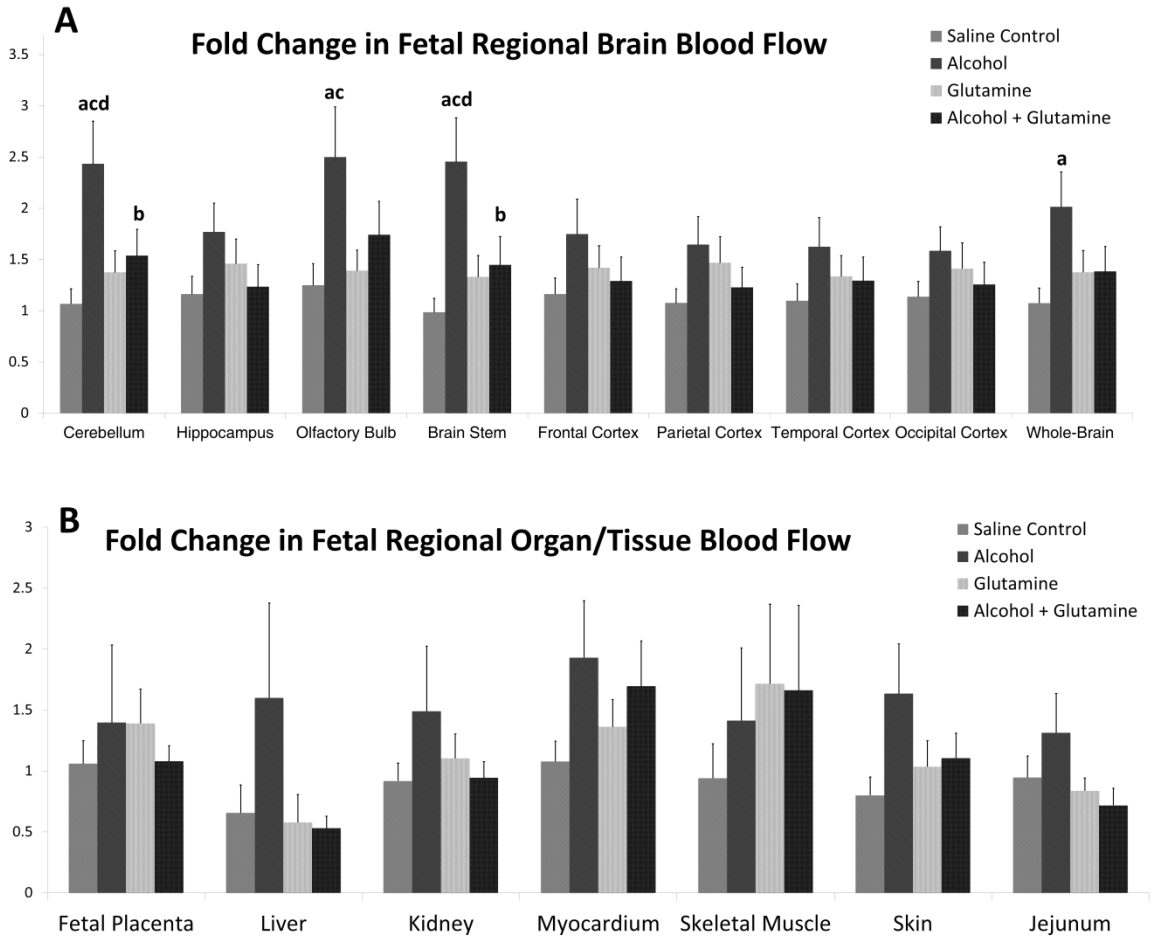

Figure 7: Fold change in fetal regional blood flow

Fetal cerebellar, olfactory bulb, brain stem and whole-brain blood flow was elevated in the alcohol group at the end of 60 min from their respective baseline (0min) on GD 120±1, compared to the control groups and maternal glutamine supplementation mitigated the alcohol-induced increase in fetal regional brain blood flow (Panel A). Fold changes in fetal regional organ/tissue blood flow at the end of 60 min from their respective baseline (0 min) did not differ among groups. **a**, **b**, **c** and/or **d** indicate statistically significant difference ($P<0.05$) compared to the saline control, alcohol, glutamine and/or alcohol+glutamine groups, respectively.

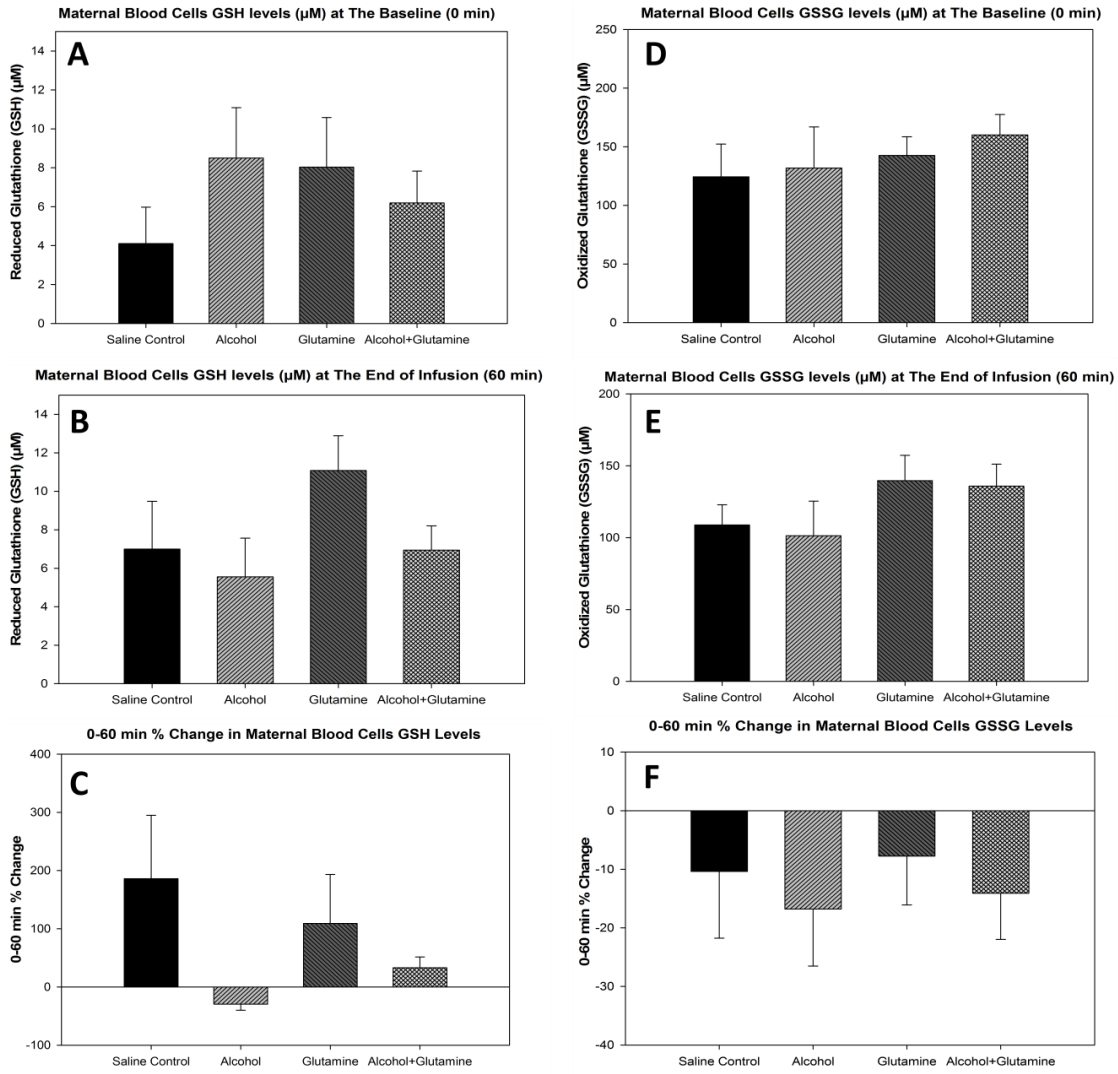

Figure 8: Reduced and oxidized glutathione levels in maternal blood cells
Levels of reduced glutathione (GSH) in maternal blood cells at 0 and 60 min (Panel A and B, respectively) and percent change between 0 to 60 min (Panel C) on GD 132. Levels of oxidized glutathione (GSSG) in maternal blood at 0 and 60 min (Panel D and E, respectively) and percent change between 0 to 60 min (Panel F) on GD 132. No statistically significant differences were observed among groups.

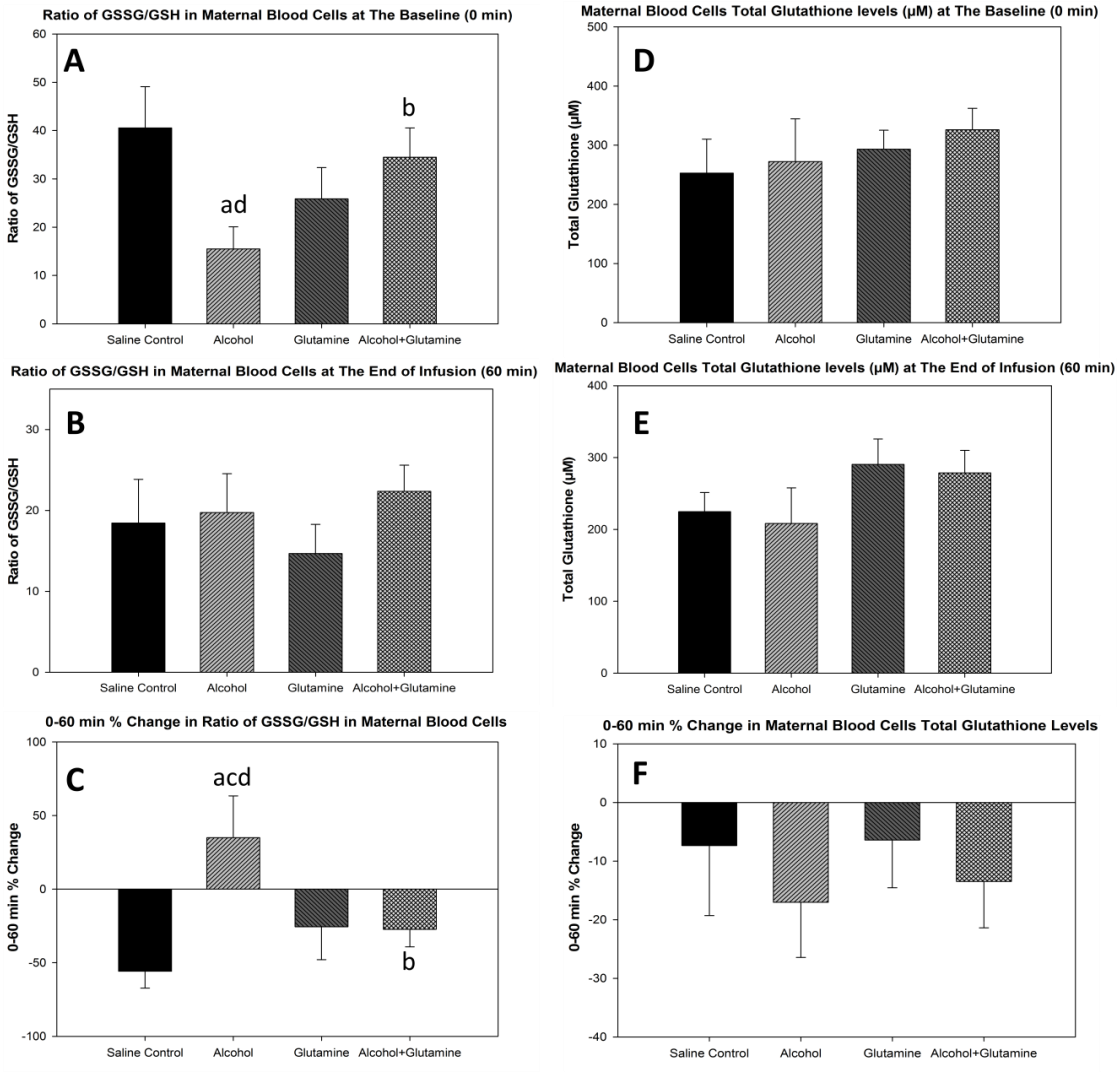

Figure 9: Ratio of oxidized to reduced glutathione and total glutathione levels in maternal blood cells

Ratio of oxidized glutathione (GSSG) to reduced glutathione (GSH) in maternal blood at 0 and 60 min (Panel A and B, respectively) and percent change between 0 to 60 min (Panel C) on GD 132. Levels of total glutathione in maternal blood at 0 and 60 min (Panel D and E, respectively) and percent change between 0 to 60 min (Panel F) on GD 132±1. **a**, **b**, **c** and/or **d** indicate statistically significant difference ($P<0.05$) compared to the saline control, alcohol, glutamine and/or alcohol+glutamine groups, respectively.

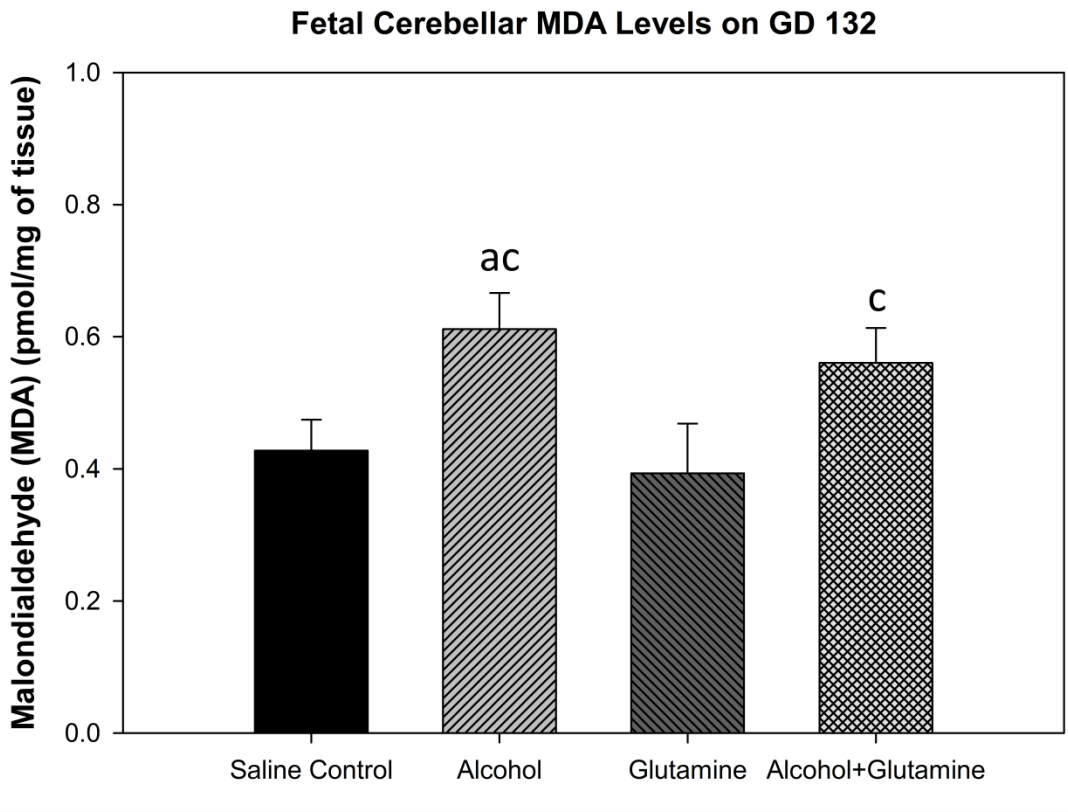

Figure 10: Levels of fetal cerebellar malondialdehyde (MDA)
Levels of fetal cerebellar MDA (pmol/mg of tissue). **a**, **b**, **c** and/or **d** indicate statistically significant difference ($P<0.05$) compared to the saline control, alcohol, glutamine and/or alcohol+glutamine groups, respectively.

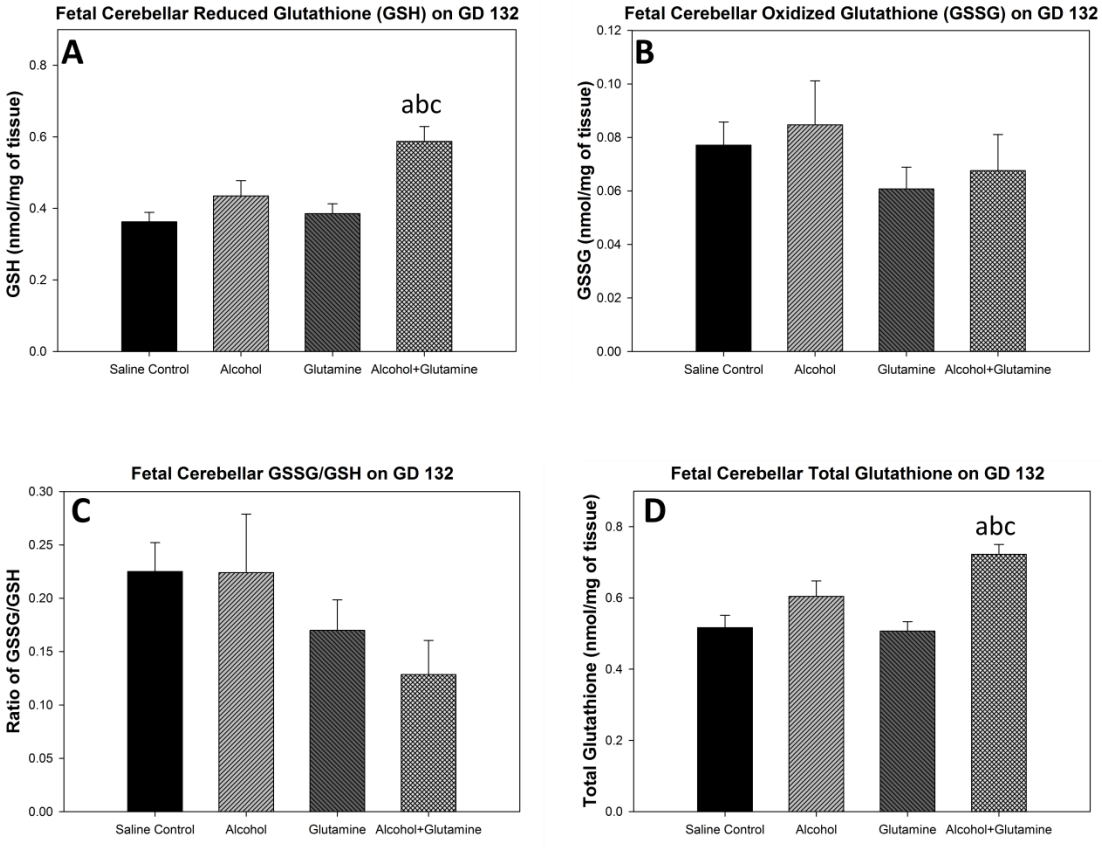

Figure 11: Levels of fetal cerebellar glutathione

Levels of fetal cerebellar reduced glutathione (GSH) (Panel A), oxidized glutathione (GSSG), ratio of GSSG/GSH (Panel C) and total glutathione (Panel D) on GD 132. **a**, **b**, **c** and/or **d** indicate statistically significant difference ($P<0.05$) compared to the saline control, alcohol, glutamine and/or alcohol+glutamine groups, respectively.

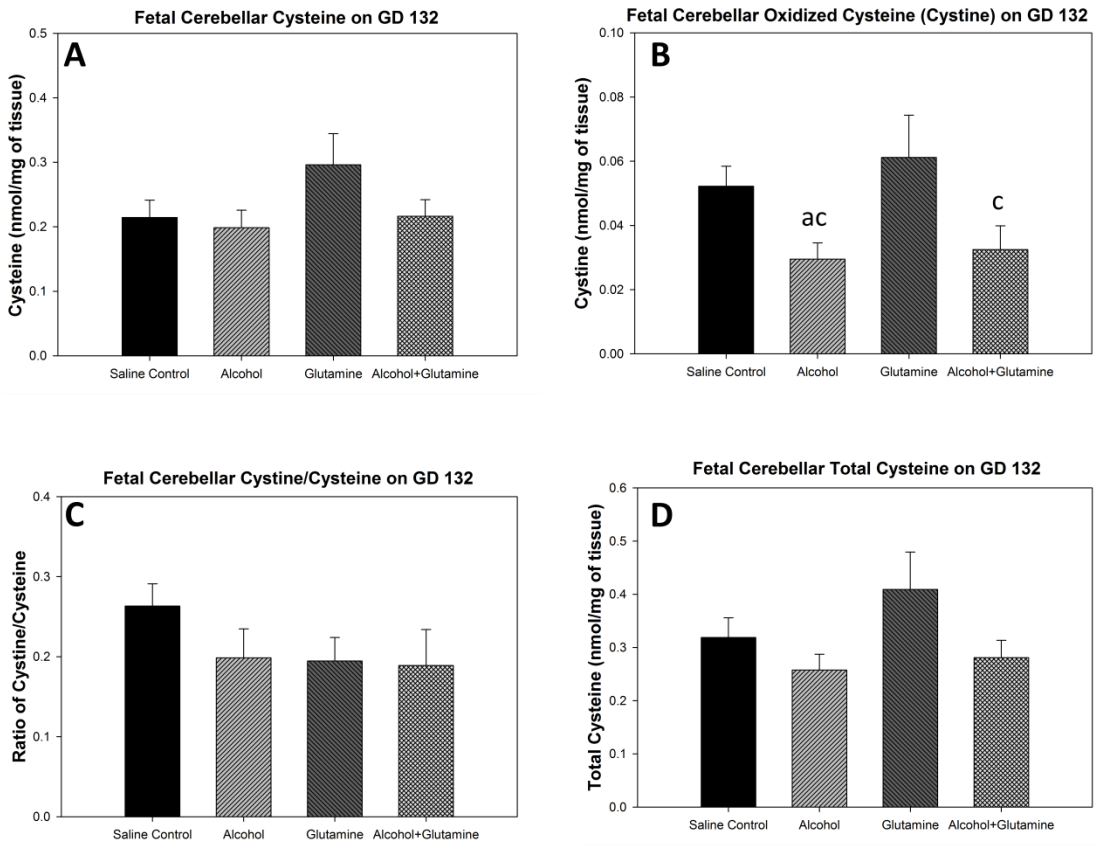

Figure 12: Levels of fetal cerebellar cysteine

Levels of fetal cerebellar free cysteine (Panel A), cystine (Panel B), ratio of cystine/cysteine (Panel C) and total cysteine (Panel D) on GD 132. **a**, **b**, **c** and/or **d** indicate statistically significant difference ($P<0.05$) compared to the saline control, alcohol, glutamine and/or alcohol+glutamine groups, respectively.

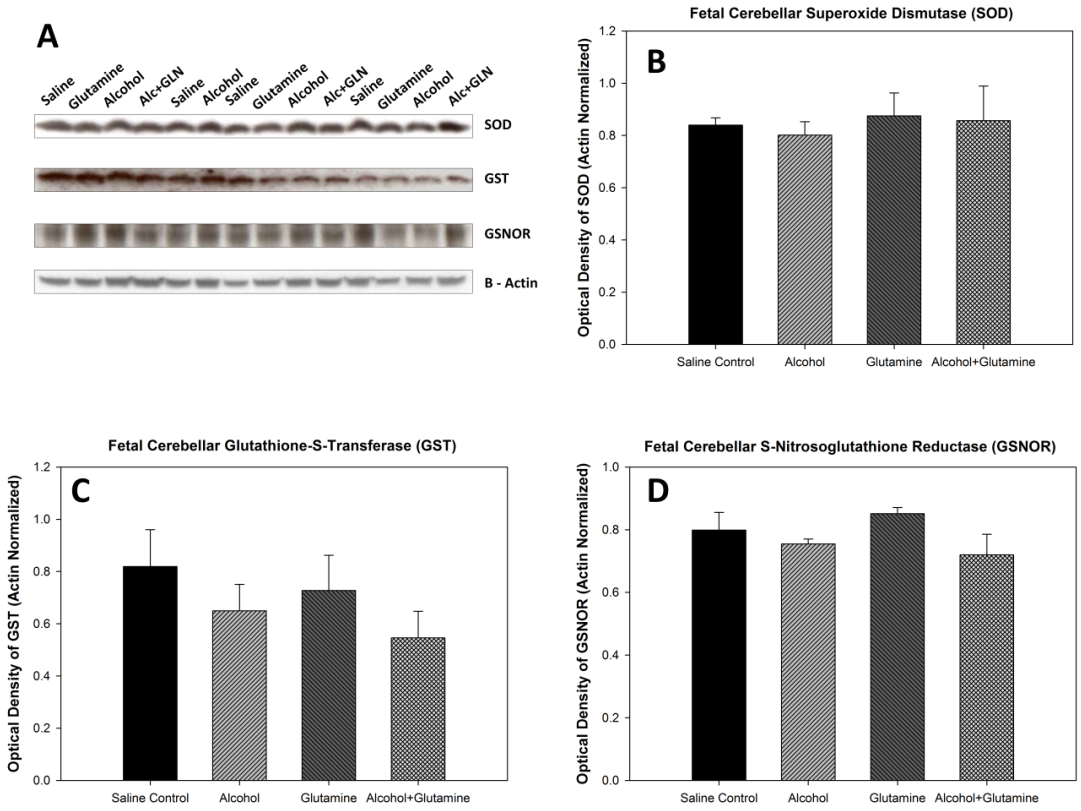

Figure 13: Expression of fetal cerebellar SOD, GST and GSNOR
Levels of fetal cerebellar Superoxide Dismutase 1 (SOD) (Panel B), S-nitroso-glutathione reductase (GSNOR) (Panel C) and glutathione transferase (GST) (Panel D) on GD 132. No statistically significant differences were observed among groups.

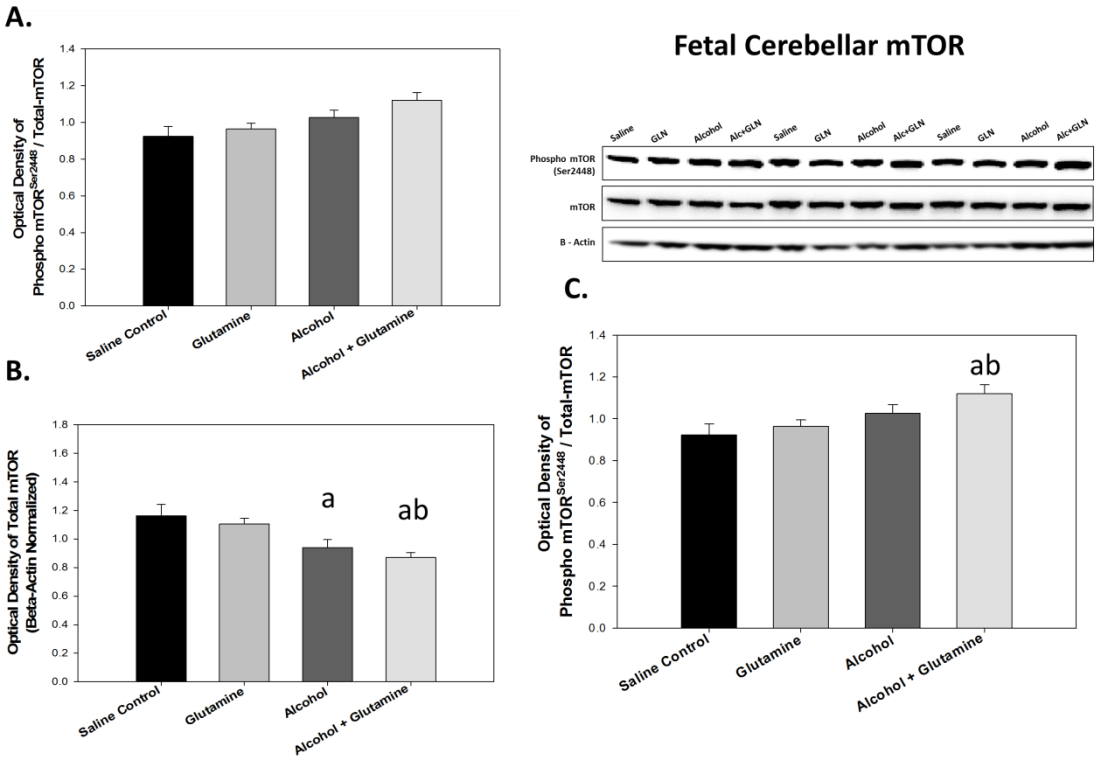

Figure 14: Fetal cerebellar mTOR

Fetal cerebellar phosphorylated mTOR (Panel A). Alcohol exposure significantly decreased levels of fetal cerebellar total mTOR in the alcohol compared to the saline control group and in the alcohol+glutamine compared to the saline control and glutamine groups (Panel B). **C.** The ratio of phosphorylated mTOR (Ser2448) to total mTOR was significantly increased in the alcohol+glutamine group compared to the saline control and glutamine groups (Panel C). **a** and **b** indicate statistically significant differences ($P<0.05$) compared to the saline control and glutamine groups, respectively.

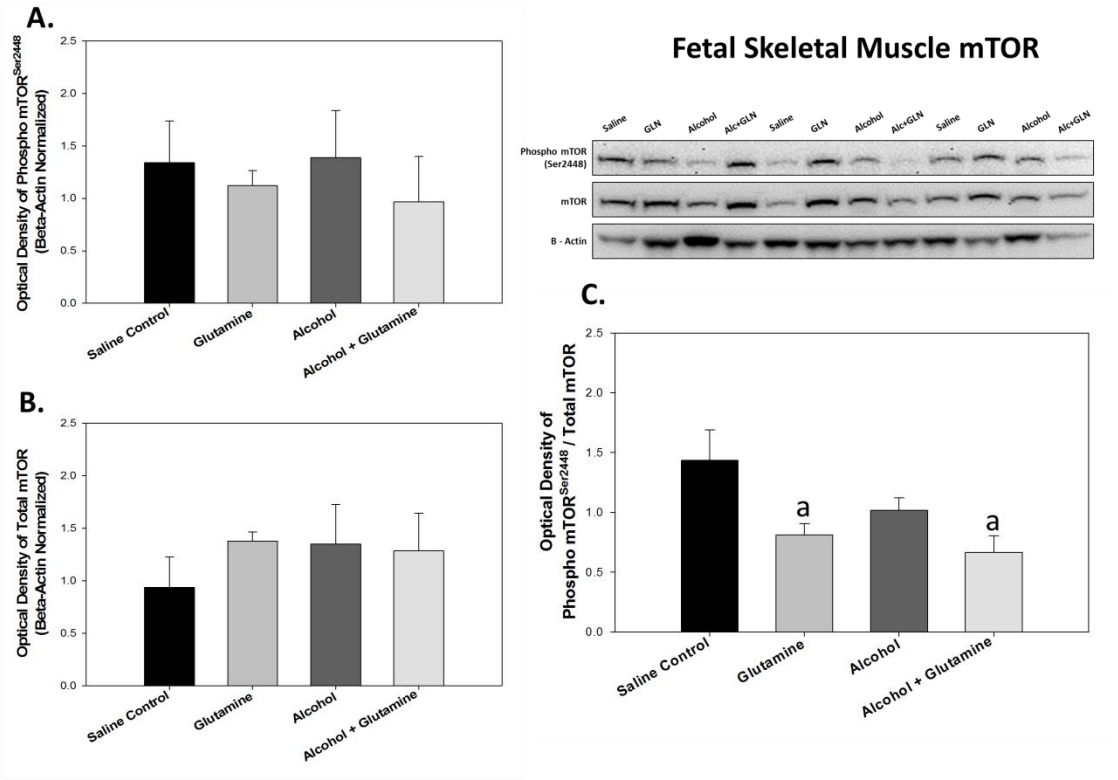

Figure 15: Fetal skeletal muscle mTOR

Fetal skeletal muscle phosphorylated mTOR (Panel A) and total mTOR (Panel B). Alcohol exposure and maternal glutamine supplementation significantly decreased the ratio of phosphorylated mTOR (Ser2448) to total mTOR in the glutamine and alcohol+glutamine groups and showed decreasing trend (p=0.078) in the alcohol group compared to the saline control group (Panel C). **a** indicates a statistically significant difference (P<0.05) in the group compared to the saline control group.

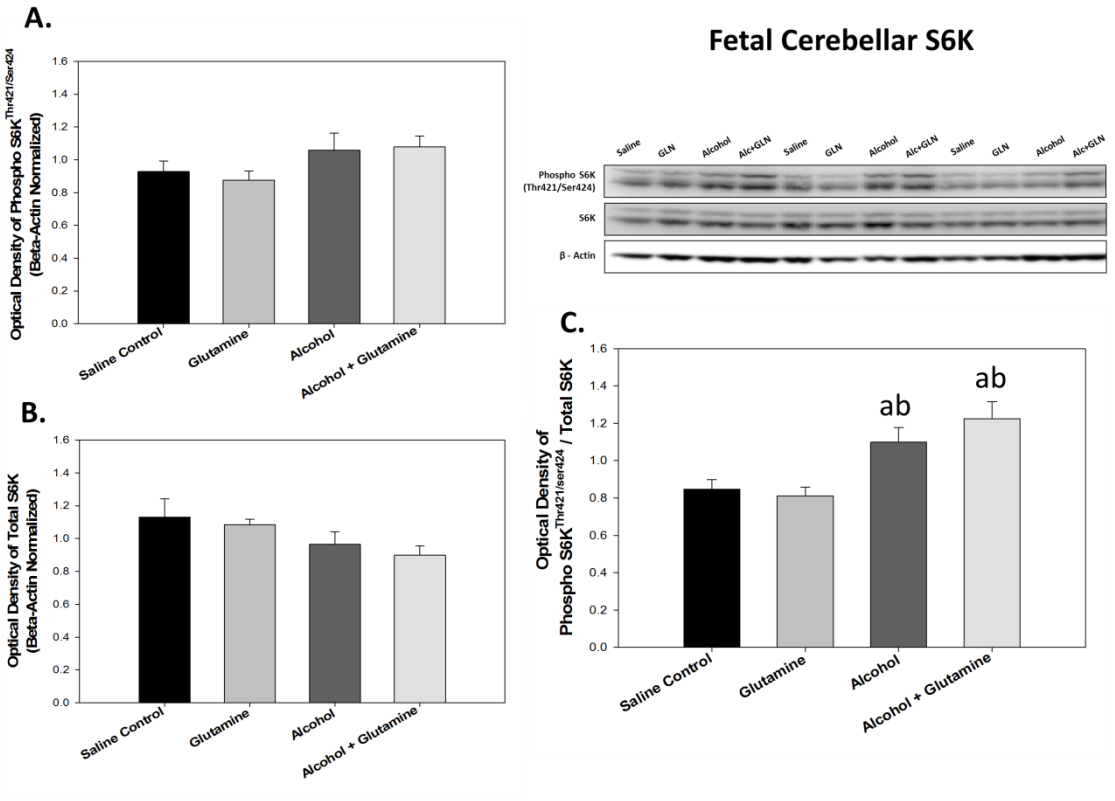

Figure 16: Fetal cerebellar S6K

Fetal cerebellar phosphorylated S6K (Panel A) and total S6K (Panel B). Alcohol exposure significantly decreased the ratio of phosphorylated S6K to total S6K in the alcohol and alcohol+glutamine group compared to the saline control and glutamine groups (Panel C). **a** and **b** indicate statistically significant differences ($P<0.05$) compared to the saline control and glutamine groups, respectively.

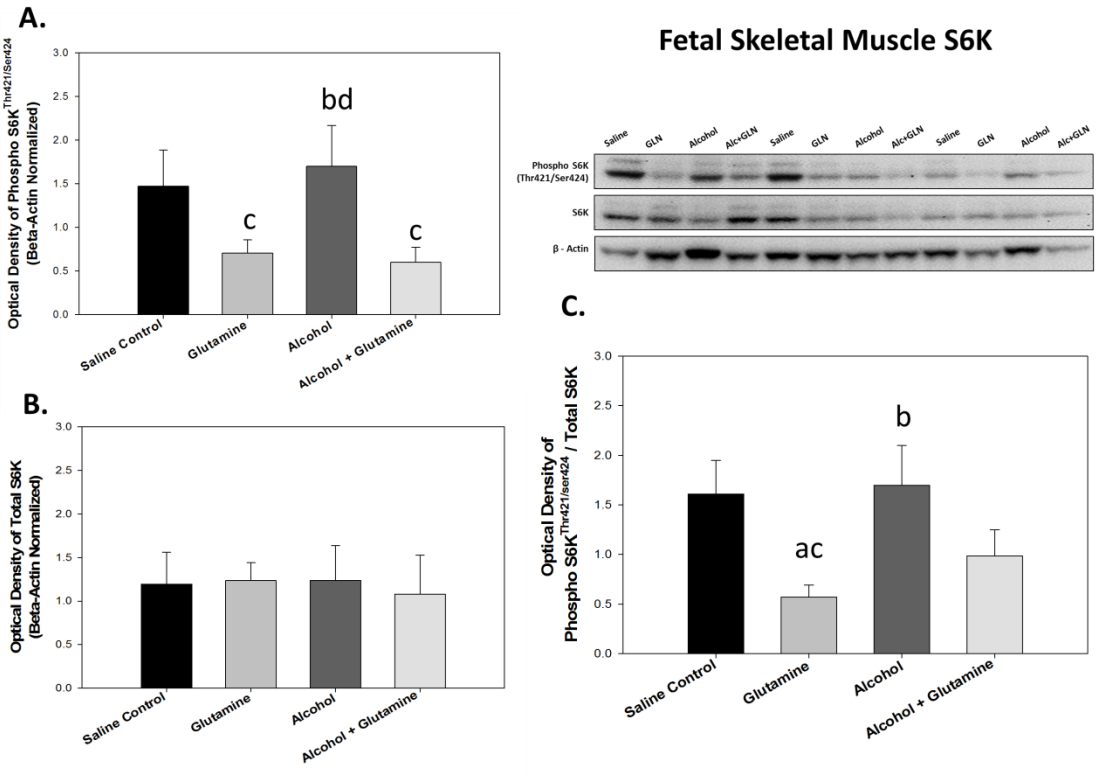

Figure 17: Fetal skeletal muscle S6K

Phosphorylated S6K expression was significantly decreased in the glutamine and alcohol+glutamine groups compared to the alcohol group (Panel A). Levels of total S6K did not differ significantly among groups (Panel B). Maternal glutamine supplementation significantly decreased the ratio of phosphorylated S6K to total S6K in the glutamine group compared to the saline control and alcohol groups (panel C). **a**, **b, c** and **d** indicate statistically significant differences ($P<0.05$) compared to the saline control, glutamine, alcohol and alcohol+glutamine groups, respectively.

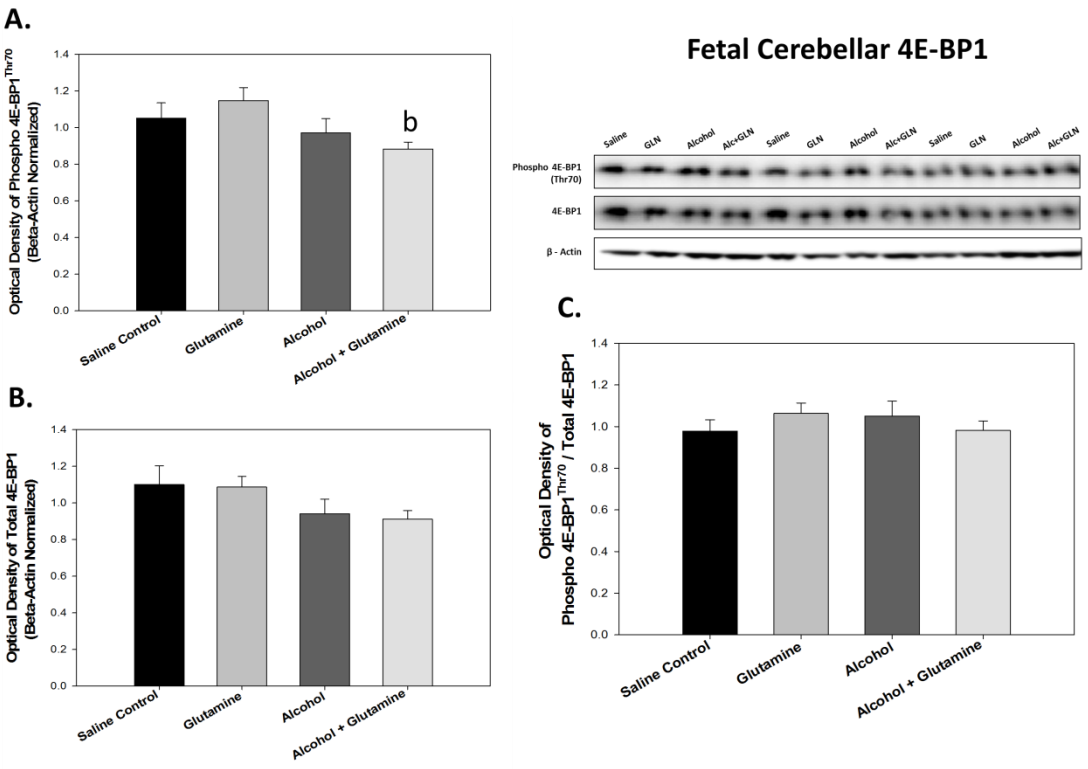

Figure 18: Fetal cerebellar 4E-BP1

Expression of phosphorylated 4E-BP1 was significantly decreased in the alcohol+glutamine group compared to the glutamine group (Panel A). Levels of total 4E-BP1 (Panel B) and ratio of phosphorylated 4E-BP1 to total 4E-BP1 (Panel C) did not differ among groups. **b** indicates a statistically significant difference ($P<0.05$) compared to the glutamine group.

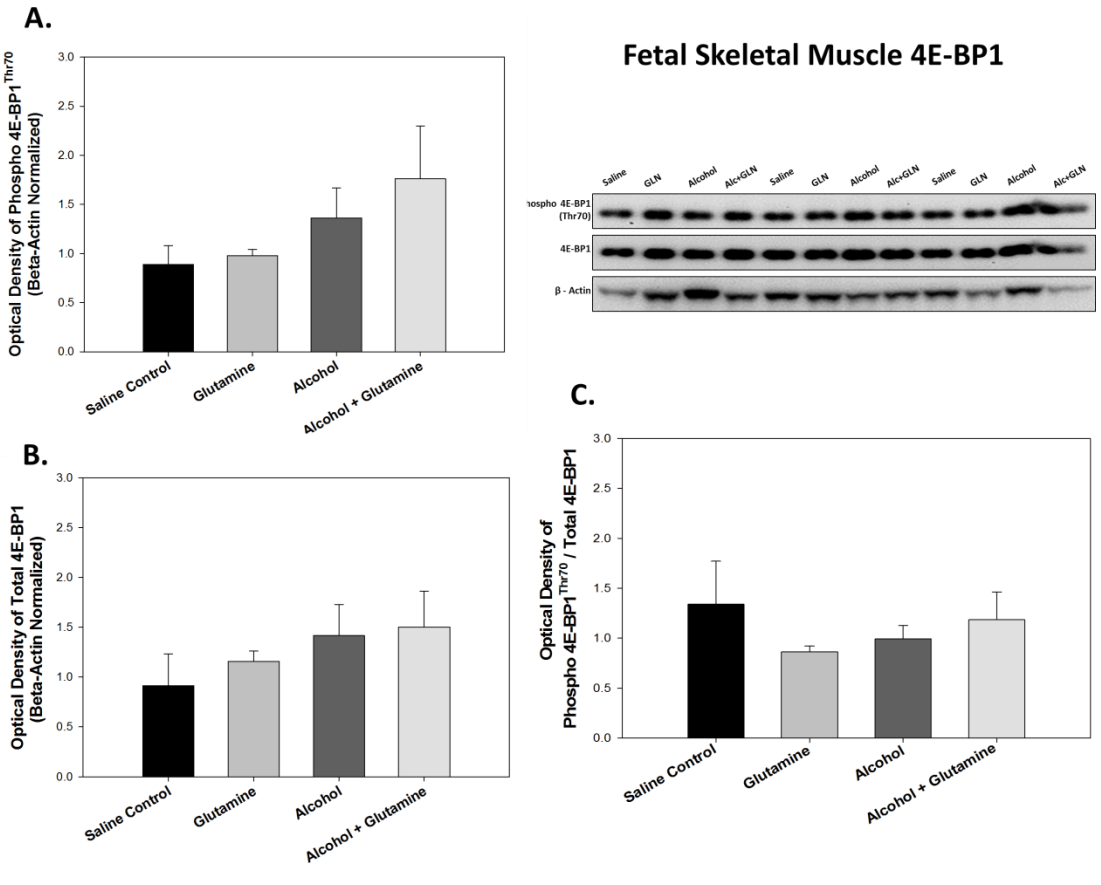

Figure 19: Fetal skeletal muscle 4E-BP1
Alcohol exposure and/or maternal glutamine supplementation did not alter the levels of phosphorylated 4E-BP1 (Panel A), total 4E-BP1 (Panel B) and ratio of phosphorylated 4E-BP1 to total 4E-BP1 (Panel C).

APPENDIX B

Table 1: Fetal body growth parameters and maternal weight on GD 132

	Saline Control	Alcohol	Glutamine	Alcohol+glutamine
Fetal Body Weight (kg)	**4.72 ± 0.20**	**4.03 ± 0.15** [acd]	**4.68 ± 0.13**	**4.73 ± 0.15**
Fetal Height (cm)	**41.33 ± 0.58**	**38.76 ± 0.66** [acd]	**41.35 ± 0.55**	**41.22 ± 0.54**
Fetal Crown-Rump length (cm)	**51.13 ± 0.79**	**47.94 ± 0.62** [ad]	**49.38 ± 0.72**	**50.48 ± 0.68**
Thoracic Girth (cm)	**34.52 ± 0.53**	**32.68 ± 0.45** [acd]	**34.48 ± 0.42**	**34.33 ± 0.34**
Abdominal Girth (cm)	33.48 ± 0.70	32.16 ± 0.56	33.94 ± 0.55	33.58 ± 0.54
Head Width (cm)	**8.21 ± 0.24**	**7.37 ± 0.12** [ac]	**7.93 ± 0.17**	**7.72 ± 0.14** [a]
Head Length (cm)	12.97 ± 0.40	12.29 ± 0.32	12.63 ± 0.21	12.69 ± 0.29
Head Circumference (cm)	20.72 ± 0.30	19.82 ± 0.36	20.46 ± 0.29	20.48 ± 0.39
Maternal Weight (kg)	84.76 ± 3.14	86.28 ± 2.62	88.91 ± 3.32	87.47 ± 2.43

Values are mean ± SEM. **a**, **b**, **c** and **d** indicate statistically significant differences ($P<0.05$) compared to the saline control, alcohol, glutamine and alcohol+glutamine groups, respectively.

Table 2: Fetal body growth parameters and maternal weight on GD 120±1

	Saline Control	Alcohol	Glutamine	Alcohol+glutamine
Fetal Body Weight (kg)	**2.85 ± 0.09**	**2.58 ± 0.05 [c]**	**3.27 ± 0.16 [a]**	**2.94 ± 0.15**
Fetal Height (cm)	34.70 ± 1.01	34.57 ± 0.62	35.45 ± 0.86	35.26 ± 0.86
Fetal Crown-Rump length (cm)	42.41 ± 0.56	43.70 ± 0.51	43.53 ± 0.67	43.50 ± 1.15
Thoracic Girth (cm)	**29.95 ± 0.42**	**28.71 ± 0.21 [c]**	**30.56 ± 0.50**	**28.97 ± 0.63 [c]**
Abdominal Girth (cm)	29.27 ± 0.58	28.68 ± 0.53	29.66 ± 0.56	28.65 ± 0.84
Head Width (cm)	6.70 ± 0.17	7.28 ± 0.21	7.31 ± 0.21	7.37 ± 0.21
Head Length (cm)	11.41 ± 0.24	11.68 ± 0.50	12.50 ± 0.25	12.00 ± 0.36
Head Circumference (cm)	18.53 ± 0.41	19.29 ± 0.36	20.09 ± 0.23	19.03 ± 0.43
Maternal Weight (kg)	82.53 ± 2.72	84.65 ± 4.93	82.36 ± 4.21	82.53 ± 3.05

Values are mean ± SEM. **a**, **b**, **c** and **d** indicate statistically significant differences ($P<0.05$) compared to the saline control, alcohol, glutamine and alcohol+glutamine groups, respectively.

Table 3: Fetal plasma amino acid levels on GD 132 (µM)

	Saline Control	Alcohol	Glutamine	Alcohol+glutamine
Aspartate (ASP)	23.98 ± 2.62	25.67 ± 2.24	30.19 ± 3.55	28.91 ± 2.14
Glutamate (GLU)	121.36 ± 22.15	126.48 ± 18.58	144.62 ± 14.85	180.55 ± 22.70
Asparagine (ASN)	**46.56 ± 5.07**	**30.69 ± 3.40** [ac]	**49.82 ± 5.09**	**36.53 ± 3.27** [c]
Serine (SER)	430.48 ± 59.87	434.48 ± 49.10	494.50 ± 50.36	512.73 ± 48.36
Glutamine (GLN)	**333.54 ± 35.35**	**203.00 ± 19.21** [acd]	**429.76 ± 41.29**	**332.36 ± 38.28** [bc]
Histidine (HIS)	**53.76 ± 6.33**	**33.59 ± 3.07** [ac]	**50.44 ± 6.07**	**41.74 ± 3.68**
Glycine (GLY)	420.75 ± 67.37	426.49 ± 57.64	485.42 ± 56.97	450.07 ± 53.33
Threonine (THR)	**216.19 ± 34.04**	**106.01 ± 12.39** [ac]	**198.58 ± 22.2**	**144.10 ± 22.63** [a]
Citrulline (CIT)	176.57 ± 22.95	152.81 ± 21.87	145.31 ± 16.96	154.52 ± 20.19
Arginine (ARG)	123.79 ± 30.69	147.55 ± 37.70	133.19 ± 23.07	128.06 ± 23.67
β -Alanine (β-ALA)	172.20 ± 29.73	160.38 ± 20.91	158.61 ± 17.64	151.48 ± 16.78
Taurine (TAU)	112.86 ± 18.44	81.37 ± 11.05	111.55 ± 16.81	105.03 ± 18.47
Alanine (ALA)	241.96 ± 31.76	227.97 ± 25.69	285.04 ± 25.56	268.03 ± 27.60
Tyrosine (TYR)	104.58 ± 17.96	82.14 ± 8.69	96.49 ± 9.47	88.44 ± 9.96
Tryptophan (TRP)	43.45 ± 6.67	45.96 ± 7.96	50.56 ± 7.84	49.44 ± 8.85
Methionine (MET)	46.25 ± 11.36	32.80 ± 7.12	48.29 ± 12.33	44.44 ± 7.90
Valine (VAL)	144.68 ± 22.71	147.95 ± 17.72	141.24 ± 16.86	178.13 ± 26.14
Phenylalanine (PHE)	91.26 ± 14.84	84.65 ± 12.22	102.47 ± 12.10	96.03 ± 12.84
Isoleucine (ILE)	50.93 ± 6.33	61.45 ± 7.17	46.19 ± 4.34	47.39 ± 4.28
Leucine (LEU)	116.80 ± 16.28	124.27 ± 14.31	110.11 ± 10.13	131.02 ± 19.58
Ornithine (ORN)	121.25 ± 22.86	146.04 ± 29.79	174.41 ± 26.73	168.56 ± 26.95
Lysine (LYS)	115.99 ± 20.31	128.48 ± 18.41	139.94 ± 18.12	157.74 ± 42.53
Branched-Chain AA (BCAA)	312.41 ± 43.46	333.68 ± 37.37	297.55 ± 29.58	354.05 ± 44.75

AA: Amino acids

Values are mean ± SEM. **a**, **b**, **c** and **d** indicate statistically significant differences ($P<0.05$) compared to the saline control, alcohol, glutamine and alcohol+glutamine groups, respectively.

Table 4: Fetal amniotic fluid amino acid levels on GD 132 (µM)

	Saline Control	Alcohol	Glutamine	Alcohol+glutamine
Aspartate (ASP)	36.02 ± 3.41	31.22 ± 2.59	33.82 ± 2.03	38.73 ± 4.42
Glutamate (GLU)	74.16 ± 8.75	57.06 ± 7.14	71.22 ± 10.59	78.43 ± 8.59
Asparagine (ASN)	**58.06 ± 9.78**	**28.77 ± 3.95 [ad]**	**46.39 ± 6.41**	**54.29 ± 9.05 [b]**
Serine (SER)	**645.84 ± 82.75**	**435.33 ± 47.26 [d]**	**700.87 ± 90.97**	**713.44 ± 65.00 [b]**
Glutamine (GLN)	**154.56 ± 43.63**	**46.42 ± 6.76 [ac]**	**147.23 ± 32.71**	**115.71 ± 27.35**
Histidine (HIS)	45.89 ± 6.90	29.49 ± 4.15	53.98 ± 12.51	42.53 ± 6.59
Glycine (GLY)	**337.36 ± 38.02**	**243.07 ± 15.66 [ad]**	**297.27 ± 25.41**	**329.19 ± 29.77 [b]**
Threonine (THR)	**114.06 ± 21.14**	**26.19 ± 4.01 [a]**	**71.58 ± 18.14**	**70.97 ± 17.69**
Citrulline (CIT)	**53.57 ± 12.45**	**8.46 ± 2.05 [a]**	**24.63 ± 6.42 [a]**	**29.55 ± 12.25**
Arginine (ARG)	138.78 ± 28.60	77.31 ± 9.93	109.85 ± 15.94	100.29 ± 13.51
β -Alanine (β-ALA)	82.70 ± 34.80	59.79 ± 13.81	124.29 ± 34.59	42.17 ± 9.76
Taurine (TAU)	247.53 ± 80.65	137.67 ± 40.05	161.83 ± 35.09	137.77 ± 32.50
Alanine (ALA)	**144.90 ± 28.14**	**78.70 ± 9.15 [ac]**	**149.07 ± 26.93**	**130.89 ± 13.76**
Tyrosine (TYR)	**96.02 ± 12.13**	**78.37 ± 6.27 [d]**	**73.19 ± 7.44**	**106.61 ± 8.17 [bc]**
Tryptophan (TRP)	37.26 ± 11.94	43.77 ± 11.36	48.28 ± 19.98	30.91 ± 12.08
Methionine (MET)	33.11 ± 10.11	32.22 ± 7.36	44.22 ± 19.20	22.51 ± 10.16
Valine (VAL)	127.40 ± 22.83	83.55 v 14.17	87.13 ± 18.78	96.36 ± 19.56
Phenylalanine (PHE)	82.57 ± 34.60	103.15 ± 31.50	77.28 ± 28.38	67.17 ± 18.39
Isoleucine (ILE)	28.44 ± 6.50	18.48 ± 2.60	19.65 ± 2.66	19.49 ± 2.87
Leucine (LEU)	**66.67 ± 11.28**	**38.56 ± 3.43 [a]**	**47.92 ± 4.94 [a]**	**53.37 ± 4.16**
Ornithine (ORN)	194.28 ± 15.63	202.69 ± 21.97	247.68 ± 24.92	192.30 ± 25.53
Lysine (LYS)	195.71 ± 40.87	127.69 ± 26.02	168.22 ± 29.41	188.06 ± 28.53
Branched-Chain AA	228.29 ± 35.70	140.59 ± 14.48	217.03 ± 62.34	174.62 ± 24.47

AA: Amino acids

Values are mean ± SEM. **a**, **b**, **c** and **d** indicate statistically significant differences ($P<0.05$) compared to the saline control, alcohol, glutamine and alcohol+glutamine groups, respectively.

Table 5: Fetal Allantoic fluid amino acid levels on GD 132 (µM)

	Saline Control	Alcohol	Glutamine	Alcohol+glutamine
Aspartate (ASP)	95.44 ± 14.45	129.01 ± 30.53	135.16 ± 20.43	74.83 ± 13.26
Glutamate (GLU)	378.95 ± 150.44	333.47 ± 80.63	308.45 ± 70.35	223.00 ± 44.64
Asparagine (ASN)	**172.96 ± 24.71**	**76.23 ± 12.21** [ac]	**173.41 ± 39.64**	**116.44 ± 23.00**
Serine (SER)	17744.81 ± 2834.31	12192.59 ± 2331.01	21036.24 ± 4709.07	15223.17 ± 2173.68
Glutamine (GLN)	919.81 ± 104.67	478.95 ± 90.93	895.69 ± 241.20	769.93 ± 112.30
Histidine (HIS)	**246.54 ± 41.73**	**112.03 ± 23.68** [a]	**212.56 ± 62.58**	**155.67 ± 25.98**
Glycine (GLY)	978.09 ± 172.24	868.96 ± 106.99	1249.41 ± 278.14	1064.33 ± 122.58
Threonine (THR)	**928.19 ± 115.86**	**400.04 ± 61.35** [a]	**661.77 ± 140.85**	**477.59 ± 66.80** [a]
Citrulline (CIT)	327.40 ± 59.16	182.60 ± 35.27	278.02 ± 66.54	223.86 ± 33.93
Arginine (ARG)	**1599.75 ± 181.12**	**1022.88 ± 171.44** [c]	**1925.92 ± 456.22**	**1108.45 ± 189.17** [c]
β -Alanine (β-ALA)	851.05 ± 212.14	597.12 ± 104.83	627.76 ± 141.20	528.50 ± 105.53
Taurine (TAU)	**5824.81 ± 741.63**	**3004.14 ± 539.78** [ac]	**5296.24 ± 926.31**	**3529.48 ± 657.09** [a]
Alanine (ALA)	1504.18 ± 305.76	822.00 ± 118.74	1275.78 ± 258.86	1128.68 ± 172.10
Tyrosine (TYR)	**622.97 ± 84.22**	**373.22 ± 45.56** [c]	**716.90 ± 123.36**	**498.32 ± 87.35**
Tryptophan (TRP)	**164.59 ± 17.30**	**99.71 ± 12.87** [c]	**253.33 ± 71.69**	**104.26 ± 16.05** [c]
Methionine (MET)	352.83 ± 57.65	225.45 ± 36.90	322.09 ± 117.16	242.60 ± 44.31
Valine (VAL)	95.87 ± 20.73	62.49 ± 9.30	96.39 ± 12.44	85.64 ± 14.47
Phenylalanine (PHE)	164.45 ± 74.25	148.69 ± 46.69	197.19 ± 72.94	89.42 ± 32.50
Isoleucine (ILE)	131.81 ± 18.62	77.05 ± 10.80	115.95 ± 27.93	89.05 ± 9.49
Leucine (LEU)	312.05 ± 34.52	190.71 ± 36.29	262.62 ± 85.58	239.18 ± 48.73
Ornithine (ORN)	**873.92 ± 183.82**	**343.37 ± 45.9** [ac]	**776.25 ± 190.07**	**506.41 ± 121.87**
Lysine (LYS)	464.1 ± 103.64	441.20 ± 57.29	451.79 ± 185.41	551.89 ± 103.29
Branched-Chain AA (BCAA)	539.73 ± 38.99	330.26 ± 47.97	479.27 ± 113.14	413.87 ± 57.19

AA: Amino acids

Values are mean ± SEM. **a**, **b**, **c** and **d** indicate statistically significant differences ($P<0.05$) compared to the saline control, alcohol, glutamine and alcohol+glutamine groups, respectively.

Table 6: Percent change in maternal arterial plasma amino acid concentrations at the end of infusion (60 min) from the baseline (0 min) on GD 120±1

	Saline Control	Alcohol	Glutamine	Alcohol+glutamine
Aspartate (ASP)	**-10.76% ± 4.21**	**-17.81% ± 11.14 [cd]**	**21.87% ± 15.3 [a]**	**12.45% ± 5.47 [b]**
Glutamate (GLU)	**-2.29% ± 5.10**	**54.17% ± 26.68 [a]**	**57.58% ± 16.72 [a]**	**92.81% ± 14.43 [a]**
Asparagine (ASN)	**-7.04% ± 6.76**	**-39.45% ± 8.97 [cd]**	**10.04% ± 12.69**	**5.42% ± 12.42 [b]**
Serine (SER)	-5.64% ± 3.22	-12.83% ± 3.25	-2.62% ± 3.79	-1.28% ± 3.79
Glutamine (GLN)	**-14.37% ± 3.14**	**-41.47% ± 8.40 [cd]**	**86.58% ± 20.16 [a]**	**35.00% ± 22.82 [abc]**
Histidine (HIS)	-7.70% ±3.02	-4.68% ± 14.43	26.27% ± 26.14	13.12% ± 13.28
Glycine (GLY)	-3.09% ± 2.69	-5.66% ± 4.91	-3.60% ± 3.90	-7.75% ± 6.49
Threonine (THR)	-15.27% ± 3.49	-28.77% ± 8.04	-17.33% ± 6.44	-38.51% ± 10.35
Citrulline (CIT)	**-15.98% ± 2.26**	**-14.84% ± 6.93 [c]**	**11.83% ± 11.70 [a]**	**-7.50% ± 4.06 [c]**
Arginine (ARG)	**3.41% ± 6.52**	**-31.45% ± 10.74 [acd]**	**-2.48% ± 7.03**	**-1.46% ± 5.10 [b]**
β -Alanine (β-ALA)	2.12% ± 4.12	-6.12% ± 11.87	5.84% ± 1.34	7.53% ± 4.13
Taurine (TAU)	**-14.01% ± 4.12**	**14.83% ± 14.55 [d]**	**31.46% ± 14.83**	**76.99% ± 26.43 [ab]**
Alanine (ALA)	-11.70% ± 5.37	-10.18% ± 6.51	-5.20% ± 8.43	-6.21% ± 10.77
Tyrosine (TYR)	-7.39% ± 1.86	-9.25% ± 4.94	-10.01% ± 1.37	-5.96% ± 2.67
Tryptophan (TRP)	-12.06% ± 9.79	-17.57% ± 13.32	-16.84% ± 10.83	-1.08% ± 4.68
Methionine (MET)	-14.28% ± 9.32	-27.36% ± 10.84	-18.25% ± 14.89	24.87% ± 38.02
Valine (VAL)	-5.91% ± 4.73	-17.47% ± 4.90	-19.21% ± 6.51	-8.13% ± 7.17
Phenylalanine (PHE)	4.11% ± 11.56	-5.45% ± 10.17	-22.10% ± 8.63	0.17% ± 23.50
Isoleucine (ILE)	-7.15% ± 5.07	-20.86% ± 6.42	-24.18% ± 5.32	-22.64% ± 7.58
Leucine (LEU)	-7.59% ± 4.36	-9.03% ± 6.87	-17.41% ± 4.09	-11.60% ± 7.60
Ornithine (ORN)	-2.69% ± 1.91	-7.59% ± 9.33	-2.00% ± 2.73	1.15% ± 1.67
Lysine (LYS)	-8.13% ± 3.84	-5.92% ± 8.19	-8.29% ± 5.71	-6.10% ± 4.34
Branched Chain AA (BCAA)	-8.18% ± 3.36	-12.73% ± 5.21	-19.76% ± 4.95	-13.13% ± 6.67

AA: Amino acids

Values are mean ± SEM. **a**, **b**, **c** and **d** indicate statistically significant differences ($P<0.05$) compared to the saline control, alcohol, glutamine and alcohol+glutamine groups, respectively.

Table 7: Percent change in fetal arterial plasma amino acid concentrations at the end of infusion (60 min) from the baseline (0 min) on GD 120±1

	Saline Control	Alcohol	Glutamine	Alcohol+glutamine
Aspartate (ASP)	4.46% ± 9.95	-0.91% ± 12.39	6.57% ± 5.85	3.17% ± 5.51
Glutamate (GLU)	**5.34% ± 4.22**	**39.45% ± 8.78 [a]**	**33.60% ± 9.67 [a]**	**20.25% ± 3.46**
Asparagine (ASN)	**-0.83% ± 3.30**	**-15.55% ± 8.21 [ac]**	**2.35% ± 2.93**	**-14.74% ± 3.19 [ac]**
Serine (SER)	**0.36% ± 1.80**	**-0.79% ± 1.67 [c]**	**7.65% ± 2.88 [a]**	**-5.86% ± 1.90 [ac]**
Glutamine (GLN)	**0.36% ± 2.64**	**-13.18% ± 6.80 [cd]**	**38.28% ± 3.63 [a]**	**23.82% ± 7.00 [ab]**
Histidine (HIS)	-3.74% ± 4.03	-6.32% ± 11.14	-3.38% ± 5.72	-4.07% ± 5.59
Glycine (GLY)	**2.07% ± 2.34**	**-12.39% ± 3.28 [ac]**	**4.23% ± 2.57**	**-14.06% ± 3.01 [ac]**
Threonine (THR)	**0.12% ± 2.29**	**-10.75% ± 3.20 [ac]**	**3.55% ± 3.30**	**-17.24% ± 2.92 [ac]**
Citrulline (CIT)	**-3.06% ± 2.09**	**1.67% ± 2.50 [d]**	**-2.97% ± 2.80**	**-8.16% ± 2.67 [b]**
Arginine (ARG)	-0.58% ± 2.04	2.17% ± 6.33	-1.11% ± 3.87	-10.05% ± 3.72
β -Alanine (β-ALA)	3.46% ± 2.11	1.21% ± 2.85	0.98% ± 3.52	-4.27% ± 4.61
Taurine (TAU)	9.32% ± 4.24	-5.94% ± 14.08	11.17% ± 4.19	-1.73% ± 6.12
Alanine (ALA)	11.77% ± 6.19	11.07% ± 5.74	18.46% ± 4.73	0.64% ± 4.89
Tyrosine (TYR)	2.82% ± 4.51	1.40% ± 5.54	-3.42% ± 3.08	-9.80% ± 7.79
Tryptophan (TRP)	-2.70% ± 11.57	-15.58% ± 7.53	-2.19% ± 5.32	-10.94% ± 6.74
Methionine (MET)	-4.77% ± 11.91	-9.45 % ± 4.99	-2.22% ± 9.43	-12..45% ± 3.30
Valine (VAL)	**3.58% ± 4.03**	**-1.08% ± 4.08**	**-4.88% ± 4.82**	**-10.41% ± 3.22 [a]**
Phenylalanine (PHE)	-3.49% ± 5.45	0.57% ± 7.90	0.47% ± 4.97	-11.44% ± 4.89
Isoleucine (ILE)	**5.91% ± 3.15**	**-12.21% ± 5.49 [a]**	**-7.29% ± 5.75**	**-19.74% ± 6.44 [a]**
Leucine (LEU)	**0.51% ± 1.74**	**-3.84% ± 4.39**	**-9.08% ± 3.21**	**-13.68% ± 4.30 [a]**
Ornithine (ORN)	**-5.00% ± 2.99**	**-12.36% ± 8.01**	**-6.69% ± 2.62**	**-17.27% ± 2.50 [ac]**
Lysine (LYS)	**-3.59% ± 2.02**	**2.81% ± 6.25 [cd]**	**-10.82% ± 3.22**	**-17.49% ± 4.50 [ab]**
Branched Chain AA (BCAA)	**6.37% ± 4.33**	**-0.76% ± 4.79**	**-6.43% ± 4.18 [a]**	**-11.98% ± 3.91 [a]**

AA: Amino acids

Values are mean ± SEM. **a**, **b**, **c** and **d** indicate statistically significant differences ($P<0.05$) compared to the saline control, alcohol, glutamine and alcohol+glutamine groups, respectively.

Table 8: Glutathione levels in maternal plasma on GD 132

Maternal Plasma		Saline Control	Alcohol	Glutamine	Alcohol + Glutamine
GSH	0 min	3.12 ± 1.51	3.61 ± 1.46	3.16 ± 1.60	2.40 ± 1.12
	60 min	4.75 ± 2.41	1.84 ± 0.62	2.61 ± 1.42	1.74 ± 0.82
	% Change	17.75% ± 40.3	-25.18% ± 15.03	-0.54% ± 13.61	-30.91% ± 8.23
GSSG	0 min	2.68 ± 1.00	1.80 ± 0.43	1.53 ± 0.29	1.49 ± 0.18
	60 min	2.73 ± 1.07	1.75 ± 0.28	1.40 ± 0.25	1.78 ± 0.31
	% Change	37.05% ± 35.70	27.13% ± 35.05	-7.34% ± 6.10	17.00% ± 9.23
GSSG/GSH	0 min	1.92 ± 0.83	0.75 ± 0.23	2.75 ± 1.23	2.15 ± 0.78
	60 min	1.27 ± 0.50	1.28 ± 0.31	3.51 ± 2.23	4.94 ± 2.56
	% Change	59.08% ± 59.39	132.98% ± 74.02	6.19% ± 24.19	85.51% ± 27.26
Total	0 min	8.47 ± 2.37	7.21 ± 2.12	5.75 ± 2.46	5.38 ± 1.27
	60 min	10.22 ± 4.45	5.35 ± 1.15	5.46 ± 2.10	5.31 ± 1.12
	% Change	8.61% ± 13.92	-1.28% ± 20.94	-2.27% ± 5.35	2.63% ± 7.95

Reduced glutathione (GSH) (µM/mg), oxidized glutathione (GSSG) (µM/mg), ratio of GSSG/GSH and total glutathione (µM/mg) levels in maternal plasma on GD 132.
Values are represented as mean ± SEM. No statistically significant differences were observed among groups.

Table 9: Cysteine levels in maternal blood cells on GD 132

Maternal Blood Cells		Saline	Alcohol	Glutamine	Alcohol + Glutamine
Cysteine	0 min	1.09 ± 0.17	0.99 ± 0.18	0.96 ± 0.14	1.01 ± 0.14
	60 min	1.16 ± 0.11	1.13 ± 0.30	0.90 ± 0.08	1.03 ± 0.12
	% Change	9.44% ± 5.76	10.48% ± 9.33	-2.30% ± 8.45	3.20% ± 5.30
Cystine	0 min	0.25 ± 0.07	0.43 ± 0.12	0.32 ± 0.12	0.41 ± 0.21
	60 min	0.38 ± 0.12	0.52 ± 0.20	0.24 ± 0.08	0.38 ± 0.14
	% Change	-13.80% ± 31.44	10.08% ± 18.53	-25.211% ± 10.56	4.99% ± 41.78
Cystine/ Cysteine	0 min	0.28 ± 0.10	0.49 ± 0.11	0.33 ± 0.09	0.35 ± 0.13
	60 min	0.37 ± 0.12	0.59 ± 0.20	0.25 ± 0.06	0.35 ± 0.09
	% Change	-24.98% ± 28.60	3.97% ± 19.61	-25.43% ± 9.41	8.30% ± 42.72
Total Cysteine	0 min	1.59 ± 0.10	1.68 ± 0.31	1.61 ± 0.32	1.83 ± 0.54
	60 min	1.75 ± 0.21	1.88 ± 0.43	1.39 ± 0.22	1.66 ± 0.34
	% Change	1.48% ± 10.05	10.19% ± 10.63	-9.51% ± 7.02	-0.19% ± 9.36

Free cysteine (µM/mg), cystine (µM/mg), ratio of cystine/cysteine and total cysteine (µM/mg) levels in maternal blood cells on GD 132.
Values are represented as mean ± SEM. No statistically significant differences were observed among groups.

Table 10: Cysteine levels in maternal plasma on GD 132

Maternal Plasma		Saline	Alcohol	Glutamine	Alcohol + Glutamine
Cysteine	0 min	7.51 ± 1.33	5.99 ± 0.82	8.34 ± 0.99	7.50 ± 0.86
	60 min	9.30 ± 1.40	8.33 ± 0.42	9.15 ± 0.91	6.79 ± 0.65
	% Change	44.47% ± 39.63	58.83% ± 31.72	11.24% ± 17.59	-6.26% ± 6.93
Cystine	0 min	34.69 ± 4.83	28.68 ± 9.57	36.36 ± 9.36	30.26 ± 5.10
	60 min	37.64 ± 6.27	38.13 ± 9.53	38.22 ± 7.74	34.85 ± 5.98
	% Change	25.34% ± 18.24	66.01% ± 36.52	2.78% ± 11.41	14.10% ± 9.21
Cystine/ Cysteine	0 min	5.23 ± 1.07	4.98 ± 1.66	4.38 ± 0.99	4.45 ± 0.99
	60 min	4.25 ± 0.60	4.71 ± 1.25	4.57 ± 1.37	5.31 ± 0.95
	% Change	-1.62% ± 13.76	7.03% ± 10.55	3.33% ± 20.03	25.46% ± 13.85
Total Cysteine	0 min	76.90 ± 9.96	63.35 ± 19.31	81.06 ± 19.07	68.03 ± 10.30
	60 min	84.57 ± 12.99	84.60 ± 18.99	85.58 ± 15.31	76.49 ± 12.15
	% Change	25.98% ± 18.63	63.22% ± 35.57	2.61% ± 9.02	11.30% ± 7.97

Free cysteine (µM/mg), cystine (µM/mg), ratio of cystine/cysteine and total cysteine (µM/mg) levels in maternal blood cells on GD 132. Values are represented as mean ± SEM. No statistically significant differences were observed among groups.