MECHANISMS UNDERLYING FETAL ALCOHOL SPECTRUM DISORDERS: OVINE MODEL

A Dissertation

by

JAYANTH RAMADOSS

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Biomedical Sciences

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Approved by:

Chair of Committee,	Timothy Cudd
Committee Members,	Wei-Jung Chen
	Jeremy Wasser
	Christopher Quick
Head of Department,	Glen Laine

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ABSTRACT

Mechanisms Underlying Fetal Alcohol Spectrum Disorders: Ovine Model. (May 2008)

Jayanth Ramadoss, B.E. (Hons.), BITS, Pilani, India; M.Sc. (Hons.), BITS, Pilani, India

Chair of Advisory Committee: Dr. Timothy Cudd

Maternal alcohol abuse during pregnancy can result in a range of structural and functional abnormalities that include lifelong physical, mental, behavioral and learning disabilities, now collectively termed as Fetal Alcohol Spectrum Disorders (FASD). The incidence of FASD is now estimated be as high as 10 per 1000 live births. Each year, 40,000 babies are born with FASD in the United States at an estimated cost of $1.4 million per individual and total cost of $6 billion. Because of the magnitude of this problem and because the incidence has not decreased in spite intensive efforts to educate women to not abuse alcohol during pregnancy, ways to prevent or mitigate the effects of prenatal alcohol exposure must be explored in addition to education. Therefore, we wished to identify the precise mechanisms by which alcohol mediates the neurodevelopmental damage in order to develop intervention/amelioration strategies.

The present study was conducted using an ovine model system. The large body mass of the ovine fetus, the longer gestation that is more similar to that of humans, and that all three trimester equivalents occur *in utero*, make the sheep an excellent model to study the effects of alcohol on the developing fetus. Our study establishes that maternal

alcohol exposure does not result in fetal cerebral hypoxia. Instead, alcohol results in hypercapnea and acidemia leading to a cascade of events in the maternal and fetal compartments that include deficits in the levels of glutamine and glutamine-related amino acids, alterations in endocrine axes, oxidative stress, alteration in cardiovascular homeostasis and fetal neuronal loss. Further, we demonstrate that inhibiting the novel two-pore domain acid sensitive potassium channel (TASK) expressed in the cerebellar granule cells and the peripheral and central chemoreceptors may prove to a be potential therapeutic strategy. Preventive strategies that are safe to use in pregnant women and that involve glutamine-related pathways are also suggested. Finally, the study also establishes the beneficial effects of moderate alcohol consumption on the fetal skeletal system.

DEDICATION

To my teachers

jñānena tu tad ajñānaḿ

yeṣāḿ nāśitam ātmanaḥ ।

teṣām āditya-vaj jñānaḿ

prakāśayati tat param ॥

“When, however, one is enlightened with the knowledge by which nescience is destroyed, then his knowledge reveals everything, as the sun lights up everything in the daytime.” <BHAGAVAD GITA 5.16>

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Finally, thanks to my mother, father, and grand mother for giving me an excellent environment to study, and for inspiring me to do a Ph.D. since I was in first grade of elementary school.

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1. INTRODUCTION

FETAL ALCOHOL SYNDROME

The term Fetal Alcohol Syndrome (FAS) was coined by scientists nearly 40 years ago to describe a pattern of birth defects found in children of mothers who consumed alcohol during pregnancy (Jones et al., 1973). Today, FAS remains the leading known preventable cause of mental retardation in the Western world (Abel and Sokol, 1991; Stratton et al., 1996); the national incidence of FAS is probably between 1 to 4.8 per 1000 live births in the United States (May and Gossage, 2001; Sampson et al., 1997; Sokol et al., 2003). The three cardinal features of FAS include a specific facial dysmorphology (midfacial hypoplasia, indistinct philtrum, thin upper lip, and short palpebral fissure), pre- and postnatal growth deficiency and varying degrees of central nervous system dysfunction (Jones et al., 1973; NIAAA, 2000; Sokol and Clarren, 1989; Stratton et al., 1996). However, the scientific and clinical reports clearly show that FAS is not the only outcome of prenatal alcohol exposure; it has been suggested that the effects of developmental alcohol exposure lie on a continuum, and is termed as Fetal Alcohol Spectrum Disorders (FASD) (Riley and McGee, 2005).

FETAL ALCOHOL SPECTRUM DISORDERS

The term Fetal Alcohol Spectrum Disorders (FASD) is an umbrella term that

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This dissertation follows the style of *Alcoholism: Clinical and Experimental Research.*

describes the range of effects that can occur in an individual whose mother drank alcohol during pregnancy (Riley and McGee, 2005; Sokol et al., 2003). Maternal alcohol abuse during pregnancy can result in a range of structural and functional abnormalities in the offspring that include lifelong physical, mental, behavioral and learning disabilities, now collectively termed Fetal Alcohol Spectrum Disorders (FASD) (Riley and McGee, 2005; Sokol et al., 2003). Despite this knowledge on the devastating outcomes of drinking alcohol during pregnancy, and in spite of massive educational campaigns about the adverse outcomes of alcohol consumption during pregnancy, the Centers for Disease Control and Prevention (CDC) reports that greater than 50% of women in the United States of childbearing age consumed alcohol in the month before their survey and that approximately 13% of these could be considered moderate or heavy drinkers (CDC, 2004). The prevalence of FASD is now estimated to be nearly 1 in 100 births (Sokol et al., 2003; May and Gossage, 2001), a number too high to deny the public health concerns related to prenatal alcohol exposure (Riley and McGee, 2005). In terms of the annual cost of FASD to the United States, each year, 40,000 babies are born with FASD in the U.S. at an estimated cost of $1.4 million per individual and total cost of $6 billion (Lupton et al., 2004).

Prenatal Alcohol Exposure and the Effects on Brain Structure

The most debilitating consequences of prenatal alcohol exposure are the ones related to brain development. The autopsy and MRI data on the anatomy of FASD

brains clearly show that the effect of alcohol on the developing brain is not uniform but varies depending on the brain region. Some of the key findings are listed below.

(1) Overall reduction of the cranial vault and a concomitant reduction in brain size (Archibald et al., 2001; Mattson et al., 1996; Riley and McGee, 2005; Swayze et al., 1997).

(2) Abnormalities in the perisylvian cortices in the parietal and temporal lobes: alcohol-exposed patients exhibit relative increases in gray matter and decreases in white matter in the perisylvian cortices of the temporal and parietal lobes (Sowell et al., 2001).

(3) Regional reductions in volume and/or tissue shape, and their displacement in space (Bookstein et al., 2002; Riley et al., 1995; Sowell et al., 2001; Sowell et al., 2002).

(4) Reduction in the volume of basal ganglia (Mattson et al., 1992).

(5) Cerebellar deficits (Autti-Ramo et al., 2002; Coulter et al., 1993).

Prenatal Alcohol and Neuronal Loss

One of the most severe forms of alcohol-induced central nervous system damage is neuronal loss. Alcohol-induced loss of neurons is dependent on some key factors:

(1) Timing of exposure: In rats, neuronal loss in different brain regions has been demonstrated to depend on the timing of alcohol exposure relative to the different phases of brain development (Bonthius et al., 1992; Cragg and Phillips, 1985; Goodlett and Eilers, 1997; Goodlett et al., 1998; Hamre and West, 1993; Livy et al., 2003; Maier and West, 2001a; Maier and West, 2003; Marcussen et al., 1994; Pauli et al., 1995; West et

al., 2001). Alcohol may act by inhibiting neurogenesis (by inhibiting important growth factors or second messenger systems that stimulate neurogenesis or preventing neuronal stem cells from responding to such stimuli), by interfering with migration, and/or by interfering with differentiation processes. For example, the developing cerebellar Purkinje cells in rats are considered to be most vulnerable during the third trimester-equivalent (Maier et al., 1999) and are susceptible to first trimester-equivalent alcohol exposure only at exceptionally high doses (6.5 g/kg) (Maier and West, 2001b) suggesting that the Purkinje cells are more vulnerable to alcohol-induced depletion during differentiation than during neurogenesis (Marcussen et al., 1994).

2. *Duration of exposure:* One study demonstrated a greater reduction in fetal cerebellar Purkinje cell number in all three-trimester alcohol exposed rats compared with that in third trimester exposed subjects, but failed to detect deficits when alcohol was administered during the first two trimester-equivalents (Maier et al., 1999).

3. *Pattern of exposure:* Binge-like drinking patterns, in which the fetus is exposed to high blood alcohol concentrations over relatively short periods of time, are particularly harmful, even if the overall alcohol amount consumed is less than those of more continuous drinking patterns (Caetano et al., 2006; Ebrahim et al., 1999; Gladstone et al., 1996; Maier and West, 2001a).

4. *The brain region:* Developmental alcohol exposure is documented to result in loss of certain neuronal cell types in specific brain regions, especially the Purkinje cells and granule cells of the cerebellum (Hamre and West, 1993; Goodlett and Eilers, 1997; West *et al.*, 2001). In fact, FASD studies conducted utilizing the rat model have shown that

the developing fetal cerebellar Purkinje cells are more vulnerable than any other cell type to alcohol-induced cell loss resulting from alcohol exposure during the third trimester-equivalent. Clinically, one of the most common abnormalities found in human prenatal alcohol exposure pathology studies has been the dysgenesis of cerebellum and/or brainstem (Clarren et al., 1978; Peiffer et al., 1979; Wisniewski et al., 1983). A significantly smaller anterior cerebellar vermis has been noted in children who have been exposed to large amounts of alcohol (Sowell et al., 1996). Recent MRI studies have suggested that the cerebellum is the most sensitive morphological indicator of prenatal alcohol exposure in children (Autti-Ramo et al., 2002).

Animal Model Systems for FASD

Apart from the clinical literature on the teratogenic effects of alcohol, much about the specific effects of gestational alcohol exposure on the fetal brain has been derived from animal model systems. Human studies on FASD include some intrinsic disadvantages (Cudd, 2005):

(1) variable consumption patterns of alcohol.

(2) unreliable self-estimates of alcohol intake by pregnant women.

(3) abuse of drugs other than alcohol.

(4) Constraints on the use of human subjects to study the teratogenic impact of alcohol.

The use of animal models circumvents many of these problems inherent in human studies. One major advantage of utilizing animal models is that they allow for

control over important maternal and environmental variables such as genetic background, nutritional status of the mother, dose and timing of alcohol exposure, as well as allowing for questions to be addressed at a more mechanistic level (Cudd, 2007). Animal studies have demonstrated that heavy alcohol exposure during development can produce deleterious effects in most of the same brain regions and organ systems that have been reported in humans with FASD. In addition, animal studies have expanded upon the gross morphological studies of humans with FASD by identifying effects at cellular (neuronal numbers) level in specific brain regions (Cudd, 2007).

The Sheep Model System

The sheep model possesses a number of advantages for the study of FASD. First, the mother and the fetus are extremely tolerant of handling surgery and chronic instrumentation. Surgically placed indwelling vascular catheters can easily be maintained for many months in adult ewe, enabling many questions in fetal physiology to be answered. The ovine fetus weighs around 4.5 kg on gestational day 133, and the adults weigh around 50 to 80 kg, comparable with that of human fetus and adult women respectively. Third, the sheep possesses a long gestation (147 days) which allows the investigator to intervene or perform experiments at specific times during gestation, and mimicking some of the human drinking patterns. Finally, the peak velocity of the fetal sheep brain growth occurs *in utero* as in humans. The maximum velocity of brain growth occurs at the time of parturition in humans, whereas it occurs postnatally in rats (Dobbing and Sands, 1973; Dobbing and Sands, 1979), requiring the assessment of the

response to alcohol administration during the third trimester-equivalent of human brain development in rats to be conducted postnatally. Therefore, to extrapolate the findings from rats to humans, one must assume that the intrauterine environment, placenta, mother and parturition play a limited role in mediating the damage (Cudd, 2005).

Mechanisms Underlying Neuronal Deficits

Delineating the mechanisms underlying FASD is very important for devising therapeutic strategies that are safe to use in pregnant women. Despite significant knowledge on the teratogenic effects of alcohol, little is known about the mechanisms involved. This is because numerous factors complicate this research (NIAAA, 2000). First, the process of development itself is enormously complex and not yet fully understood. Second, no single mechanism can account for the spectrum of deficits observed in FASD. Third, alcohol-mediated deficits depend on several variables like the timing of exposure, duration of exposure, frequency of consumption, amount of drinking (blood alcohol concentration), the mother's health, and the genetic makeup of the mother and the fetus (NIAAA, 2000). Despite these difficulties, scientists have described a few plausible mechanisms that can account for the FASD phenotypes:

(1) Interference with the cell-cell interaction: The brain abnormalities seen in children with FASD can resemble those of children with L1 gene mutation (Chen et al., 2001). Ethanol disrupts L1-mediated cell-cell adhesion (Charness et al., 1994) and this action is prevented by 1-Octanol that antagonizes ethanol inhibition of L1-mediated cell–cell adhesion (Chen et al., 2003). However, the underlying mechanisms are largely unclear

and speculative. This mechanism was further tested by administering NAPVSIPQ (NAP) and SALLRSIPA (SAL) (active peptide fragments of two neuroprotective proteins) that potently and completely antagonized the inhibition of L1 adhesion (Wilkemeyer et al., 2002). In contrast to these observations, others have reported that ethanol does not inhibit the adhesive activity of drosophila neuroglia or human L1 in drosophila s2 tissue culture cells (Vallejo et al., 1997). Clearly, more work is needed to understand this mechanism.

(2) Alteration of intracellular calcium levels: Alcohol has been shown to alter calcium levels through glutamate receptor mediated mechanisms (Gruol et al., 1996). However, underlying mechanisms are still not very clear.

(3) Formation of oxygen free radicals: It is now widely known that prenatal alcohol exposure increases reactive oxygen species levels as well as reduces endogenous anti-oxidant levels leading to developmental oxidative stress (Cohen-Kerem and Koren, 2003; Goodlett et al., 2005).

(4) Alteration in the levels of growth factor and/or the cell's ability to respond to these factors: Maternal plasma insulin-like growth factor 1 (IGF-1) concentrations are reduced by 51% in response to alcohol, with a 20% reduction in hepatic IGF-1 mRNA levels (Breese and Sonntag, 1995), and these factors are responsible for cell proliferation and survival in the developing fetus (NIAAA, 2000).

(5) Alteration in neurotransmitter systems: Two neurotransmitter systems are particular targets of alcohol exposure: the glutamate and the serotonin system. Prenatal alcohol exposure studies have repeatedly demonstrated NMDA (glutamate receptor) receptor

down regulation during the third trimester-equivalent of human brain development (NIAAA, 2000), and modest NMDA receptor activation is essential as it has trophic effects, and has permissive effect on the action of other neurotrophic factors (Tsai and Coyle, 1998). Others have demonstrated that very early prenatal exposure to alcohol can lead to delay in the development of the serotonergic system, leading to developmental deficits.

(6) Alteration in gene expression and protein synthesis

(7) Alteration in maternal-fetal interaction and/or maternal conditions: This is a largely overlooked area as the peak velocity of brain growth in the most widely used animal model (the rat) occurs postnatally, requiring the investigator to perform experiments on a neonatal model. This approach entirely ignores the role played by the mother, the placenta, maternal-fetal interactions, and parturition in mediating the teratogenic effects of alcohol.

(8) Alteration in nutrient levels: Alcohol could lead to fetal undernutrition by at least three possible ways: reduced maternal dietary intake (Schenker et al., 1990), impaired intestinal and/or placental transport of specific nutrients (Fisher et al., 1981; Henderson et al., 1981; Lin, 1981; Polache et al., 1996), and altered maternal and/or fetal metabolism and compartmentalization of nutrients (Schenker et al., 1990).

Gaps in Knowledge

Clearly, despite the vast clinical and experimental FAS literature, surprisingly little is known of the mechanisms of damage. Alcohol is a ubiquitous drug that may

affect the brain at any or all stages of development, but its primary disruptive mechanism during development is unknown. Whether deficits are produced by a single or by multiple cascading mechanisms, is clearly a complex issue that needs to be addressed (Abel and Hannigan, 1995). Until this issue is resolved, it will be extremely difficult to develop successful therapeutic intervention strategies. This report addresses some of the important questions regarding some key mechanisms underlying Fetal Alcohol Spectrum Disorders using the ovine model where all three trimester-equivalents occur *in utero*. Section 2 provides the background and sets the stage for the remainder of the report. This section shows that the experimental evidence supporting alcohol-induced cerebral hypoxia as a mechanism of FASD is largely indirect, and that moderate alcohol does not result in fetal hypoxia; rather it results in maternal and fetal hypercapnea and acidemia. This section also discusses the selective vulnerability of the fetal cerebellum in response to third trimester-equivalent alcohol exposure. Sections 3, 4, and 5 examine the critical windows of vulnerability during development and establish the similarities between the structural deficits observed in the ovine model system and the humans. Section 6 evaluates the hypothesis that maternal alcohol acidosis could lead to nutritional deficits. Section 7 discusses the role of acidemia in fetal neuronal loss and potential therapeutic treatments that completely prevent alcohol-induced neuronal loss. Sections 8 and 9 discuss the effects of alcohol on fetal and maternal skeletal system. Section 10 lists the conclusions of the report.

2. BACKGROUND: FETAL ALCOHOL AND HYPOXIA*

INTRODUCTION

Ethanol has long been recognized as a neuroteratogen that can cause deficits in motor coordination, behavior, cognitive and executive function, now referred to as Fetal Alcohol Spectrum Disorder (FASD) (Sokol et al., 2003). Numerous hypotheses have been proposed to account for these actions, of which cerebral hypoxia is one of the most popular. The experimental evidence supporting ethanol-induced cerebral hypoxia as a mechanism of FASD is largely indirect. For example, both hypoxia and ethanol exposure during development induce similar craniofacial and neuronal deficits (Aitken and Schiff, 1986; Bronsky et al., 1986). Direct evidence that cerebral hypoxia may be a mechanism by which prenatal ethanol exposure causes FASDs was supplied by a report that an intravenous bolus of ethanol in fetal primates resulted in fetal hypoxemia (Mukherjee and Hodgen, 1982). However, the ethanol dose (3.0 g/kg) and administration paradigm (intravenous bolus over two minutes) used in that study resulted in exceptionally high blood ethanol concentrations (BECs) of ~500 mg/dl. Additionally, that study was performed in anesthetized animals: anesthesia may have impeded the normal protective cardiovascular homeostatic responses to the actions of ethanol. Other studies have demonstrated that an acute (Falconer, 1990; Reynolds et al., 1996;

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*Reprinted with permission from Parnell SE, Ramadoss J, Delp MD, Ramsey MW, Chen WJ, West JR, Cudd TA (2007) Chronic ethanol increases fetal cerebral blood flow specific to the ethanol-sensitive cerebellum under normoxaemic, hypercapnic and acidaemic conditions: ovine model. Exp Physiol 92(5):933-43, Copyright [2007] by Blackwell Publishing Ltd.

Richardson et al., 1985; Smith et al., 1989a) or a chronic ethanol exposure (1-3 g/kg) in fetal sheep during all of (Cudd et al., 2001b) or during the last week (Patrick et al., 1985) of the third trimester equivalent (Dobbing and Sands, 1979) does not result in hypoxemia.

Taken together, these reports suggest that, at least with moderate doses, ethanol does not create hypoxemia during the third trimester. However, ethanol may still induce fetal cerebral hypoxia by reducing cerebral blood flow (CBF). Mann and coworkers first demonstrated that an acute ethanol exposure in near-term sheep fetus under general anesthesia resulted in increases in CBF (Mann et al., 1975). Gleason and Hotchkiss (1992) utilized mid-gestation sheep and found no decreases in fetal CBF. Richardson and coworkers (1985), again using near-term fetal sheep, observed decreased CBF, oxidative metabolism, and brain activity in response to an acute dose of ethanol (1 g/kg). However, no one had investigated the correlation between regional CBF changes and regional differences in neuronal vulnerability in response to chronic ethanol exposure. Animal studies conducted utilizing the rat model had repeatedly shown that the teratogenic effects of ethanol are region-specific (Bonthius et al., 1992; Goodlett and Eilers, 1997; Livy et al., 2003). Developmental third trimester-equivalent ethanol exposure is documented to result in loss of certain neuronal cell types in specific brain regions, especially the Purkinje and granule cells of the cerebellum (Hamre and West, 1993; Goodlett and Eilers, 1997; West et al., 2001). Therefore, we hypothesized that

repeated ethanol exposures, 3 days in succession per week (a common binge drinking paradigm) (Caetano et al., 2006; Ebrahim et al., 1999; Gladstone et al., 1996) throughout the third trimester would: 1) result in a change in fetal CBF in response to acute ethanol challenge following chronic binge exposure, 2) induce changes in CBF that are region specific, occurring preferentially in brain regions that are vulnerable to ethanol-induced injury and, 3) result in fetal normoxemia. The measurement of regional CBF was performed using radiolabelled microsphere technique and the assessment of regional differences in neuronal loss associated with chronic binge ethanol exposure was conducted using a stereological cell counting technique. These experiments were performed in the sheep fetus during a period of high velocity brain growth like that during the third trimester in humans (Cudd, 2005), a period when it is known that the brain is sensitive to ethanol mediated damage (Maier et al., 1999).

METHODS

Subjects

The experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University. Suffolk ewes, aged 2-6 years of age, were mated and pregnancies of known date of conception were confirmed as previously described (Ramadoss et al., 2006a). The day of mating (the day that ewes were marked by the ram) was designated as gestational day (GD) 0. Ewes were maintained in shaded outdoor pens with herdmates from before mating until GD 90. On GD 90, the ewes were relocated to an environmentally regulated facility (22ºC and a 12:12 light/dark cycle) where they remained for the duration of the experiments. Animals in all treatment groups were fed 2 kg/day of a "complete" ration (Sheep and Goat Pellet, Producers Cooperative, Bryan, TX). All animals consumed all of the feed offered.

Treatment Groups

For the CBF study, four treatment groups were used: a low ethanol dose, 0.75 g/kg (E075), a moderate ethanol dose, 1.75 g/kg (E175), a saline control (SC) group and a normal control (NC) group. The potential confounding effect from the ethanol or saline infusion procedure was controlled for by the NC group; this group was subjected to surgery, instrumentation and microsphere injection but no ethanol or saline infusion. On gestational day 109, the beginning of the third trimester equivalent in this species, an intravenous catheter (16 ga., 5.25 inch Angiocath™, Becton Dickinson, Sandy, UT) was

placed into the jugular vein in the ethanol and saline infusion subjects. Ethanol or saline was administered intravenously (IV) over a one-hour period via a peristaltic pump (Masterflex, Model 7014-20, Cole-Parmer, Niles, IL). The ethanol solution was prepared by adding 95% ethanol to sterile 0.9% saline to achieve a 40% w/v ethanol solution. Solutions were prepared under aseptic conditions and were administered through a 0.2 μm bacteriostatic filter. This ethanol solution was then administered according to the weight of the ewe in order to achieve the appropriate ethanol dosage. The saline control group (SC) received an infusion of physiological saline (0.9%) that was equal in volume to the 1.75 g/kg ethanol infusion. Pumps were calibrated before each infusion. The pregnant ewes received ethanol or saline in a binge-like paradigm (modeling weekend only binge drinking), consisting of three consecutive days of exposure followed by four days without exposure. This pattern was repeated throughout the third trimester equivalent (GD 109-132; term is 147 days) for a total of 12 ethanol exposures. There were 8 fetuses in the E175 group, 7 fetuses in the E075 group, 8 fetuses in the SC group and 4 fetuses in the NC group. All pregnancies were monotocus except for one twin pregnancy in each of the NC and E175 groups and two twin pregnancies in the SC group. In cases of twins, only one fetus was studied.

The assessment of regional differences in neuronal loss in response to ethanol was conducted utilizing 2 separate treatment groups: an ethanol group (E175) (n = 5) and a saline control group (Control) (n = 5). The fetuses were not instrumented in this study; instead only the ewes underwent surgery to chronically implant femoral arterial and venous polyvinyl chloride catheters (0.050” inner diameter, 0.090” outer diameter)

on gestational day 102 as previously described (Cudd et al., 2001b). The ethanol exposure regimen was similar to the CBF study. At the end of the experiment, on gestational day 133, the fetuses were removed from the uterus and perfused with saline followed by cold fixative solution containing 1.25% paraformaldehyde and 3% glutaraldehyde in phosphate buffer (pH, 7.4). The brains were removed and stored in additional fixative until processed for stereological cell counting.

Fetal Surgery

Surgery was performed between GD 119 and 120 to implant chronic indwelling catheters as previously described (Cudd et al., 2001b). In brief, a ventral midline laparotomy was performed and the uterus and fetal membranes were incised. A catheter (0.030" inner diameter, 0.050" outer diameter polyvinyl chloride) was passed from the femoral artery into the inferior aorta. A catheter (0.040" inner diameter, 0.070" outer diameter polyvinyl chloride) was passed from the saphenous vein into the inferior vena cava. A catheter (0.030" inner diameter, 0.050" outer diameter polyvinyl chloride) was passed from the brachial artery into the brachiocephalic trunk. A catheter (0.050" inner diameter, 0.090" outer diameter polyvinyl chloride) was fixed to the exterior of the fetus for the purpose of measuring amniotic cavity pressure. Catheters were passed through the flank of the ewe and were stored in a pouch attached to the skin.

Blood Flow Measurement

Blood flow was measured on gestational day 132 at 0, 1, and 2 hours relative to the beginning of the final ethanol infusion using the radioactive microsphere technique (Rudolph and Heymann, 1967). Radiolabelled microspheres, 15 μm in diameter (Perkin-Elmer, Boston, MA), 20 μl at a concentration of 25,000/μl, were suspended in a solution of physiological saline and 0.01% Tween-80. The microsphere suspensions were subjected to 10 min of sonication followed by 1 min of vortexing and then were injected as a 1 ml bolus into the fetal inferior vena cava via saphenous vein catheter. A separate radioisotope was used for each time point (^{57}Co, ^{46}Sc and ^{85}Sr). The reference blood samples were withdrawn from the catheter in the femoral artery and brachial artery by precision syringe pump (Harvard Apparatus, Holliston, MA) at 2 ml/min. After the final microsphere injection, the ewes were euthanized using an intravenous injection of sodium pentobarbital (75 mg/kg). The uterus was removed from the ewe, the fetus was exteriorized and 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 10 regions: frontal cortex, parietal cortex, temporal cortex, occipital cortex, subcortex, cerebellum, white matter, midbrain, pons and the medulla. The samples were weighed, placed into tubes and activity was measured (Packard Auto-Gamma 5780). Blood flow in each tissue sample was calculated from the reference sample activity, withdrawal rate and the tissue activity and weight. After calculation of blood flows, paired organs (e.g. left and right kidneys) were compared to ensure proper mixing of the microspheres with blood . In no cases was there evidence of incomplete mixing.

Blood Gases and Blood Ethanol Concentration Measurements

PaO_2, $PaCO_2$, pH and hematocrit were measured from blood samples taken from the fetal arterial catheter at 0, 1, and 2 hours relative to the beginning of the final ethanol infusion. Maternal BECs were measured using head-space gas chromatography (Varian, model #3900, Palo Alto, CA) as described before (West et al., 2001). Blood gases and pH were measured using a blood gas analyzer (ABL 5; Radiometer, Westlake, OH). Arterial PO_2 values were temperature corrected to 40ºC (Lotgering et al., 1983). Fetal arterial oxygen saturation was calculated after accounting for Bohr effect as described before (Battaglia et al., 1970). Cerebral oxygen delivery was then determined from the whole brain blood flow and arterial oxygen content measures.

Systemic Hemodynamic Measurements

Fetal heart rate and mean arterial pressure (MAP) were measured from the beginning of the final one hour ethanol infusion until one hour after the infusion. Phasic blood pressure was sampled at 60 hz beginning at time 0 for 2 hrs from the abdominal aorta via the femoral artery catheter using a strain gauge transducer (Isotec, Quest Medical, Allen, TX), analog-digital converter (DAQ Card-AI-16XE-50, National Instruments, Austin, TX) and a notebook computer and heart rate and mean arterial pressure were calculated (Labview, National Instruments, Austin, TX). Biventricular output was calculated by summing the blood flows for each gross fetal region (brain, heart, kidney etc.).

Brain Tissue Processing and Assessment of Regional Differences in Neuronal Loss

The cerebellum was first dissected from the rest of the brain. The right olfactory bulb was removed at the junction of the caudal olfactory bulb and olfactory tract. The brain was then divided parasagittally and the right hippocampus was removed. The cerebellum (embedded in 4% agar, and cut sagittally into five slabs), the olfactory bulb and the hippocampus were dehydrated through increasing concentrations of ethanol (70, 95, 100%) and then infiltrated with increasing concentrations of infiltration solution (25, 50, 75, 100% methyl methacrylate; Historesin^{TM} Embedding Kit, Leica, Wetzlar, Germany). The tissue was embedded in a solution containing 1 ml dimethyl sulfoxide (hardener) per 15 ml of 100% infiltration solution and allowed to harden. After hardening, the cerebellar tissue was cut into 30 μm sagittal sections and the others coronally by using a microtome (model RM2255, Leica, Nussloch, Germany). Sections were saved, mounted on a glass slide, stained with cresyl violet, and coverslipped. The total number of fetal cerebellar Purkinje cells, hippocampal pyramidal cells (CA1 and CA2/3), dentate gyrus granule cells, and the olfactory bulb mitral cells were determined using unbiased stereological cell counting techniques as described before (West et al., 2001; Ramadoss et al., 2007a). In brief, the Nikon (Garden City, NY) Optiphot microscope used in this study had a 40X objective lens for the cerebellar measurement and a 60X lens for the olfactory and hippocampal tissues with a 1.4 numerical aperture condenser. The microscope had a motor-driven stage to move within the x and y axes and an attached microcator to measure the z axis. The image was transferred to a personal computer (Millenium, Micron, Boise, ID) via a color video camera (model

2040, Jai, Copenhagen, Denmark). The reference volume was estimated using Cavalieri's Principle and was calculated by the equation $V_{ref} = \Sigma p_i \times A(p_i) \times t$ where Σp_i is the total number of points (p_i) counted, $A(p_i)$ is the known area associated with each point, and t is the known distance between two serial sections counted. The GRID® software provided templates of points in various arrays that were used in point counting for reference volume estimation. The neuronal cell density was determined by following the optical disector method, which was calculated using the formula $N_v = \Sigma Q / (\Sigma disector \times A(fr) \times h)$ where ΣQ is the sum of the neurons counted from each disector frame, $\Sigma disector$ is the sum of the number of disector frames counted, A(fr) is the known area associated with each disector frame, and h is the known distance between two disector planes. The placement of the disector frames was determined by the GRID® software in a random manner. The estimated total number of neurons in the brain structures were then calculated by multiplying the reference volume of the brain structure and the numerical density of cells within this reference volume as described before (West et al., 2001).

Statistical Analyses

The data from the NC and SC groups for each dependent variable were compared and no differences were detected. Therefore, these groups were combined to create a single control group. Fetal biventricular output, mean arterial pressure, total peripheral resistance, blood gases, arterial pH and organ blood flows were all analyzed using a two-way ANOVA with treatment as a between factor and time as a within factor. When appropriate (when significance of a factor or of interaction was established by the initial analysis), an analysis of simple effect was performed for each time point using one-way ANOVA. Further pair-wise comparisons were performed when appropriate using Fisher's protected least significant difference (PLSD). For heart rate and mean arterial pressure, one min mean values were utilized for statistical analysis. Neuronal cell number data was analyzed using one-way ANOVA with "treatment" as the sole independent variable followed by protected Fishers LSD tests. The α level was established *a priori* at $p < 0.05$ for all analyses.

RESULTS

The experimental design of the CBF study included two control groups, one receiving a saline infusion of equivalent volume and rate as the higher ethanol dose group (E175) and a second control group (normal control), receiving neither ethanol nor saline infusion. The data from the NC and SC groups for each dependent variable were compared and in no cases were there differences between these two groups and were therefore combined. For example, a two-ANOVA conducted on the whole brain blood flow (ml/min/100 g) did not yield a significant main effect of treatment group or time or an interaction between the two factors. Mean $\pm$ SEM values at 0, 1, and 2 hours were 139 $\pm$ 19, 120 $\pm$ 22, and 168 $\pm$ 19 in the normal control group and 165 $\pm$ 16, 153 $\pm$ 17, and 122 $\pm$ 16 in the pair-fed saline control group respectively.

Blood Ethanol Concentration

The mean maternal blood ethanol concentration (BEC) measured at 0, 1 and 2 hours in the E075 and E175 groups peaked at 1 hr which coincided with the end of the infusion period. Peak BECs were 185 $\pm$ 26 mg/dl and 85 $\pm$ 6 mg/dl in the E175 and E075 groups respectively.

Fetal Blood Gases

The fetal arterial pH (pH_a) was significantly different among treatment groups (Figure A-1). The pH_a at one hour in the E175 group was significantly lower compared with that in the control and E075 groups. However, pH_a in the E075 group did not

change significantly compared with that in the control. The arterial partial pressure of carbon dioxide (P_aCO_2) was similarly different among groups. At one hour, the P_aCO_2 was significantly elevated in the E175 group compared to the control and E075 groups. The E075 group was also different from the control group at one hour. There were no significant differences among treatment groups or over time in hematocrit or arterial partial pressure of oxygen (P_aO_2).

Fetal Systemic Hemodynamics

Fetal mean arterial pressure at one hour was significantly lower in the E175 group compared with that in the control and the E075 groups (Figure A-2). However, the E075 group was not different from the control group. The decrease in the mean arterial pressure in the E175 group was also accompanied by an increase in fetal heart rate. The heart rate was significantly elevated in the E175 group at one hour compared to the control and the E075 groups; heart rate in the lower dose group was not different from that in the control group.

Biventricular output was calculated from reference samples taken from the brachial artery and femoral artery. Because these measures were not different at the 0^{th} hour and because the number of subjects from which brachial artery reference samples were collected was lower compared with the femoral artery (brachial artery samples: control group, n = 6; E075 group, n = 4; E175 group, n = 4; femoral artery samples: control group, n = 12; E075 group, n = 7; E175 group, n = 8) as a result of sample withdrawal failures, we only used the blood flows calculated from reference samples

taken from femoral artery for all flow analyses. Biventricular output was significantly elevated at one hour in the E175 group compared with that in the control group, whereas the lower dose group was not different from the control group. Total peripheral resistance, derived from the mean arterial pressure and the biventricular output measures, was significantly decreased in the E175 group at one hour compared to the control group.

Fetal Cerebral Blood Flow

The fetal whole brain blood flow at one hour was significantly higher (~ 37 %) in the E175 group compared with that in the control group (Figure A-3). Analysis of regional brain blood flow identified cerebellar blood flow in the E175 group to be significantly higher (~ 44 %) at one hour compared to the control group, whereas the E075 group was not different from the control group (Figure A-4). Further, the blood flow at one hour in the E175 group trended numerically upward in pons, medulla, subcortex, frontal cortex and temporal cortex, contributing to the observed overall increase in whole brain blood flow but the response was not statistically significant.

Cerebral Oxygen Delivery

Oxygen delivery (ml of O_2 /100 ml of blood / 100 g brain tissue / min) was increased by 38% in the E175 group at 1 hour compared with that in the control group. Despite a mild decrease in oxygen content due to Bohr effect at the peak hour in the E175 group compared with that in the control group, there was an overall increase in oxygen delivery due to a significant increase in whole brain blood flow.

Regional Differences in Cell Loss Associated with Chronic Binge Ethanol Exposure

The E175 group produced a significant loss of fetal cerebellar Purkinje cells compared with that in the control group (Figure A-5). However, no such differences between the ethanol treated group and the controls were observed in other brain tissues like the hippocampus and the olfactory bulb. The hippocampal pyramidal cells in the CA1 and CA2/3 field, the granule cells of the dentate gyrus as well as the mitral cells of the olfactory bulb were not different between treatment groups.

DISCUSSION

Ethanol Results in Fetal Normoxemia, Hypercapnea, and Acidemia

In this study, we hypothesized that fetal ethanol exposure would not result in fetal hypoxemia. Consistent with previous findings, the mean P_aO_2 did not decrease in response to fetal ethanol exposure (Cudd et al., 2001b; Richardson et al., 1985). Even though we previously reported mild maternal hypoxemia in response to ethanol, it has been documented that the fetus is particularly adapted to protect itself from diminished oxygen supply. Fetal differences from the adult are higher hemoglobin concentration, left-shifted oxygen-hemoglobin saturation curve, and higher cardiac output per unit body weight (Thornburg and Morton, 1994). Further, in spite of a mild decrease in fetal arterial oxygen content due to the Bohr effect, the overall cerebral oxygen delivery was actually increased in response to the moderate ethanol dose (E175) at one hour as the brain blood flow was significantly increased. This finding coupled with reports by others that fetal hemoglobin concentration is not altered in response to ethanol (Gleason and Hotchkiss, 1992; Richardson et al., 1985) provide convincing evidence that oxygen delivery to the brain is not compromised during the third trimester equivalent at these BECs. Further, previous studies have demonstrated in rats during the third trimester equivalent that ethanol doses capable of reducing fetal cerebellar Purkinje cell number do not alter global brain intracellular high-energy phosphate concentrations (Cudd et al., 2000), an indication that brain oxygenation is not altered and that ethanol does not mediate hypoxic, anemic, ischemic or histotoxic hypoxia. The present study is the first to demonstrate that cerebral oxygen delivery is not compromised in response to chronic

binge ethanol exposure. Therefore, factors other than hypoxia are responsible for the fetal neurodevelopmental damage in response to moderate doses of ethanol exposure during the third trimester equivalent of human fetal development**.**

The present study also demonstrates an increase in fetal P_aCO_2 and a fall in fetal arterial pH following one hour infusion of ethanol. This finding is in agreement with a number of previous reports from our laboratory showing that with each bout of maternal ethanol, the mother and the fetus experience transient hypercapnea and acidemia (Cudd et al., 2001b; Ramadoss et al., 2007b; West et al., 2001).

Ethanol Alters Fetal Systemic Hemodynamics

The fetal mean arterial pressure was significantly decreased at one hour in response to the moderate ethanol dose (E175). This reduction in arterial pressure was due to an ethanol-induced decrease in the total peripheral resistance as biventricular output was elevated. The observed reduction in fetal total peripheral resistance and increase in biventricular output was also accompanied by a simultaneous elevation in fetal heart rate.

Moderate doses of ethanol have been demonstrated to modulate vascular endothelial cell functions through a variety of mechanisms. First, ethanol may exert most of its vasorelaxant effects via the nitric oxide system and/or dilatory prostanoids induced by fetal hypercapnea and acidemia (Iadecola and Zhang, 1994; Leffler et al., 1994). In the present study, the decrease in total peripheral resistance was significant only in the E175 group and not in the E075 group. This is likely because the fall in fetal

pH_a was significant only in the E175 group. Second, chronic ethanol may increase nitric oxide activity by increasing plasma levels of estradiol 17β, either by increasing the activity of aromatase or by decreasing the metabolism of estrogen (Chung, 1990; Turner and Sibonga, 2001). Third, ethanol may decrease vascular tone by inducing nitric oxide release resulting from ethanol mediated elevation in extracellular adenosine levels (Kurth and Wagerle, 1992; Nagy et al., 1990; Orrego et al., 1988; Rekik et al., 2002). Thus, adenosine, CO_2, pH and perhaps other metabolites could reduce the tone of small downstream arterioles which, in turn, could act to produce a lower intraluminal pressure in intermediate-size arterioles upstream that possess a strong myogenic response (Kuo et al., 1992). The myogenic dilation of these vessels would further reduce vascular resistance and subsequently increase flow. The increased flow could initiate flow-induced dilation in larger arterioles upstream, which are characterized by a less potent myogenic response but possess a dominant flow-dependent vasodilatory mechanism (Kuo et al., 1992). Therefore, ethanol may recruit metabolic, myogenic and flow mediated mechanisms to decrease the total peripheral resistance.

We speculate that the increase in fetal heart rate observed in this study was in response to an ethanol mediated fall in the mean arterial pressure via baroreflexes. Even though acute fetal hypotension stimulates a reflex bradycardia, mild hypotension has been shown to produce elevated heart rate in fetal sheep (Iwamoto and Rudolph, 1981; Wood et al., 1989; Wood and Tong, 1999).

Biventricular output was assessed directly by summing the individual organ blood flows (ml/min) rather than by calculating cardiac output from injected isotope

counts and counts from the reference blood withdrawal sample (Ishise et al., 1980); the small numbers of microspheres that adhere to the inside of the catheters during the infusion can result in an overestimation of cardiac output using this latter method (Delp et al., 1991; Delp et al., 1998). The method of summing tissue blood flows to assess biventricular output consists of tissue blood flows throughout the body, including that to the adrenals, brain, heart, lungs, kidneys, spleen, thyroids, thymus and skeletal muscle. The placental blood flow was not examined in this study due to difficulty in separating fetal and maternal components but the placental vasculature does not autoregulate and is considered a passive bed (Thornburg and Morton, 1994). Biventricular output to these tissues at this stage of gestation in fetal sheep has been previously reported to be ~ 360 ml/min, as measured using radiolabelled microspheres and by electromagnetic flow probe (Rudolph and Heymann, 1967, 1970). This is in close agreement with that obtained in the present study under baseline conditions (SC, 331 ml/min; E075, 310 ml/min; E175, 406 ml/min). Thus, the baseline biventricular output in this study is similar to that reported in classic chronically prepared fetal ovine studies.

Ethanol Alters Cerebral Blood Flow

The baseline fetal whole brain blood flow (around 150 ml/min/100 g of brain tissue) in this study is in agreement with others utilizing near-term fetal sheep (Gratton et al., 1996; Purves and James, 1969; Rudolph and Heymann, 1970). In this study, all blood flows were calculated from reference samples taken from the femoral artery. While brachial artery samples would have provided better absolute values for cerebral

blood flow, all conclusions in this study were based on changes from baseline rather than absolute flows, which could not have differed regardless of the location of the withdrawal site (Edelstone and Rudolph, 1979; Reuss et al., 1981).

We hypothesized that an acute ethanol infusion following the chronic exposure would alter CBF, and we found that whole brain blood flow increased in response to the moderate ethanol dose. Mann and coworkers also found increased CBF in response to an acute ethanol exposure in fetal sheep on a similar day of gestation (Mann et al., 1975). However, their study was acute and was conducted on exteriorized fetal sheep under general anesthesia, conditions that could have affected the responses to ethanol. Gleason and Hotchkiss, 1992 reported that a single ethanol (1 g/kg) infusion did not alter CBF in 92 day gestation fetal sheep. However, developmental differences in the fetal vascular response to maternal ethanol consumption would be expected as for example, NOS expression varies not only regionally but also temporally during development (Northington et al., 1997). Therefore, experiments performed at different times of gestation may not necessarily produce the same findings for a specific dependent variable. Common to these studies is the finding that CBF is not decreased in response to maternal ethanol consumption. Richardson and coworkers (1985) observed decreased CBF, oxidative metabolism, and brain activity in response to an acute dose of ethanol (1 g/kg) in near term sheep. However, their subsequent study demonstrated that an ethanol infusion following repeated ethanol exposures did not decrease fetal brain activity, due to development of tolerance (Smith et al., 1989b). Though CBF was not measured in this latter study, we predict that CBF would not have fallen due to close coupling of

brain metabolism and CBF (Seisjo, 1978), an assertion that is also supported by the present findings. Our results further contribute to the existing literature by demonstrating that CBF is increased in response to repeated moderate ethanol exposures throughout the third trimester-equivalent, and that fetal cerebrovascular response to acute and chronic ethanol consumption are very different. The dosing paradigm used in the present study models a common drinking pattern in humans and therefore better represents what occurs in the fetus of women who binge drink during pregnancy. Furthermore, the increase in CBF observed in this study could be part of a vicious cycle; ethanol mediated increase in CBF would further increase the brain ethanol delivery, exacerbating the neuroteratogenic effects.

Ethanol Alters CBF in Regions Vulnerable to Prenatal Ethanol Exposure

We also hypothesized that ethanol induced alterations in brain blood flow would be region specific, occurring preferentially in ethanol sensitive regions. The baseline blood flows in the different brain regions in this study were comparable to those reported in a previous study utilizing near-term fetal sheep (Richardson et al., 1985). Fetal cerebellar blood flow was increased significantly at one hour in response to the moderate ethanol dose (E175) (Figure A-5) while other brain regions, the subcortex, frontal cortex, temporal cortex, pons and medulla, showed a similar but non-significant pattern of change in response to ethanol, contributing to an increase in total brain blood flow. Interestingly, this finding is strongly correlated with the cell count data. A significant reduction in the cerebellar Purkinje cell number in response to ethanol was observed but

cell loss was not observed in other brain tissues that were examined. Animal studies have repeatedly demonstrated differences in regional and temporal vulnerability of the brain to developmental ethanol exposure (Bonthius et al., 1992; Goodlett and Eilers, 1997; Livy et al., 2003). Developmental ethanol exposure is documented to result in loss of certain neuronal cell types in specific brain regions, especially the Purkinje cells and granule cells of the cerebellum (Hamre and West, 1993; Goodlett and Eilers, 1997; West et al., 2001). In fact, the developing fetal cerebellar Purkinje cells are more vulnerable than any other cell type to ethanol induced cell loss resulting from ethanol exposure during the third trimester-equivalent. In the present study, CBF changes were specific to brain regions that were found to be especially sensitive to developmental ethanol exposure, namely the fetal cerebellum, demonstrating that the physiological response to ethanol may not be the same in different vascular beds within the fetal brain. Vasculature in the different regions of the developing fetal brain may behave differently because of altered release of local factors from differentially vulnerable neurons or glia. We propose that fetal hypercapnea and acidemia may be an important cause of increases in CBF as well as for the neuronal damage; however, more studies need to be conducted to identify specific mechanisms that contribute to the observed regional differences.

SUMMARY

We conclude that chronic prenatal ethanol exposure in sheep, during a period of brain development equivalent to third trimester in humans, produces regional differences in neuronal loss, decreases in mean arterial pressure and fetal total peripheral resistance and increases in CBF selectively in the ethanol-sensitive cerebellum, where neuronal loss was observed. Our study demonstrates that the fetal cerebrovascular response to chronic ethanol exposure is very different from acute ethanol exposure responses reported by others. These hemodynamic alterations in the fetus are not accompanied by fetal hypoxia, but rather by hypercapnea and acidemia. We also propose that ethanol mediated fetal hypercapnea and acidemia may be the principle mechanism behind alterations in CBF as well as in producing fetal Purkinje cell loss. Thus, this study provides a strong causal link among prenatal ethanol exposure, increases in CBF and fetal brain injury in the absence of hypoxia.

3. THREE TRIMESTER ALCOHOL EFFECTS ON THE CEREBELLUM*

INTRODUCTION

Maternal alcohol consumption during pregnancy can result in a range of debilitating outcomes in the offspring that include physical, mental, behavioral and/or learning disabilities, now termed Fetal Alcohol Spectrum Disorder (FASD). The incidence of FASD has not decreased in spite of significant efforts in educating women to not drink during pregnancy (Institute of Medicine, 1996; MCH data report, 2003; NIAAA, 2000). Women who drink while pregnant are at a high risk of giving birth to children with neurodevelopmental disorders, and the affected children may display abnormal development of the cerebellum (Archibald et al., 2001; Sowell et al., 1996; Swayze et al., 1997). Cerebellar Purkinje cell loss and disorientation has been noted in an infant whose mother binged during the first trimester of pregnancy (Coulter et al., 1993). Although no quantitative data regarding cerebellar neuronal loss are currently available from human FAS studies, extensive data derived from animal models have shown that prenatal alcohol exposure causes significant reduction in the cerebellar Purkinje cell number (Chen et al., 2003). In rats, the reduction in neurons in different brain regions has been demonstrated to depend on the timing of alcohol exposure relative to different phases of brain development (Bonthius et al., 1992; Cragg and

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Phillips, 1985; Goodlett and Eilers, 1997; Goodlett et al., 1998; Hamre and West, 1993; Livy et al., 2003; Maier and West, 2001a; Maier and West, 2003; Marcussen et al., 1994; Pauli et al., 1995; West et al., 2001). The third trimester-equivalent has been found to be the most critical period of vulnerability in the rat model (Goodlett and Eilers, 1997; Goodlett et al., 1998; Hamre and West 1993; Maier et al., 1999). However, the third trimester-equivalent with regard to brain development occurs after parturition in the rat. Therefore, to extrapolate these findings to humans, one must assume that the intrauterine environment, placenta, mother and parturition do not play an important role in mediating the damage (Cudd et al., 2005). Previously, we reported that third trimester equivalent alcohol exposure in an ovine model, where cerebellar development occurs prenatally to the extent that it does in humans, produces a 25% reduction in fetal cerebellar Purkinje cell number (West et al., 2001). In the present study, we hypothesized that all three trimester prenatal alcohol exposure *in utero* would result in a greater magnitude change in cerebellar Purkinje cell number compared with third trimester exposure only, by disrupting events occurring antecedent to the third trimester and thus enhancing the neuronal damage. While this question has been previously addressed in the rat model, it has not been addressed in an animal model where all three trimester equivalents occur in utero.

In addition to the determination of critical windows of vulnerability, we examined potential mechanisms that may be involved in the fetal brain injury seen in FASD. A previous report from our laboratory has shown that with each bout of maternal alcohol abuse, the mother and the fetus experienced transient hypercapnea and acidemia (Cudd et al., 2001b), and acidosis has been documented to cause cell losses both in neurons and glial cells (Goldman et al., 1989; Kraig et al., 1987; Nedergaard et al., 1991; Staub et al., 1990). Therefore, a secondary goal in this study was to assess the changes in maternal blood gases and pH following a binge-like alcohol exposure throughout gestation. We employed a weekend binge pattern, a drinking pattern common in women who use alcohol during pregnancy (Caetano et al., 2006; Cudd et al., 2001a; Ebrahim et al., 1999; Gladstone et al., 1996; Maier and West, 2001a) and who are more likely to refrain compared to everyday drinkers, if convinced that drinking poses a hazard to their baby.

METHODS

Animals and Breeding

The experimental procedures employed in this study were approved by the University Laboratory Animal Care Committee at Texas A&M University. Suffolk ewes (aged 2 to 6 years) maintained on coastal Bermuda grass pasture and supplemented with alfalfa hay were bred under controlled conditions. Time dated pregnancies were achieved by controlling the estrous cycle through the use of progesterone impregnated vaginal implants (EAZI-BREED™, CIDR®, Pharmacia & Upjohn Ltd., Auckland New Zealand). Implants were removed 11 days after placement at which time prostaglandin $F_{2\alpha}$ (20 mg; LUTALYSE®, Pharmacia & Upjohn, Kalamazoo MI) was intramuscularly administered. The following day, ewes were placed with a ram fitted with a marking harness for a period of 24 hours. Marked ewes were assessed ultrasonographically on 25, 60 and 90 days to confirm pregnancy. On day 4 of gestation, the saline control and alcohol group subjects were moved into individual pens but were able to have visual contact with herdmates in the adjacent pens at all times. Conditions of constant temperature (22 ^{0}C) and fixed light dark cycle (12:12) were maintained. Once confined, the saline and alcohol treatment group subjects received 2 kg/day of a complete ration (Sheep and Goat Pellet, Producers Cooperative Association, Bryan, TX). Daily feed consumption was monitored; subjects in the alcohol and saline treatment groups consumed all of the food offered. Subjects in the normal control group remained in the pens with herdmates throughout the study. Subjects in this group were offered, as a

group, an equivalent amount of feed compared to subjects in the saline and alcohol control groups. However, individual feed consumption was not monitored.

Alcohol Dosing Protocol

In the alcohol treatment group (alcohol dosage of 1.75 g/kg body weight), and the saline control (0.9% saline) groups, infusion solutions were delivered intravenously by peristaltic pump (Masterflex, model 7014-20, Cole parmer, Niles IL) through a 0.2 µm bacteriostatic filter. Pumps were calibrated before infusion. Alcohol infusions were 40% W/V in sterile saline administered over one hour. Alcohol was administered on three consecutive days beginning on day 4 of gestation followed by 4 days without alcohol.

Experiment Protocol

Details of the experiment protocol have been described earlier (Ramadoss et al., 2006a). In brief, the experiments began on day 4 and were terminated on day 133 of gestation. On gestational day 4, an intravenous catheter (16 gauge, 5.25 in Angiocath™ Becton Dickinson, Sandy, UT) was placed percutaneously into the jugular vein. On the day of infusions, ewes were connected to the infusion pump by 0830 hr and alcohol was infused continuously over 1 hr. On gestational day 42, after pregnancy was confirmed ultrasonographically, the ewes underwent surgery to chronically implant femoral arterial and venous vascular access ports (V-A-P™, Model CP 47P, Access Technologies, Skokie IL). The ewes were not surgically instrumented until after the first trimester to

avoid early embryonic losses. Infusions were then given through the venous port and blood was sampled from the arterial port.

Blood was drawn from the jugular vein catheter on gestational days 6 and 40 and from femoral artery catheter on days 90 and 132 every 30 min for 2 hr and at 6 and 24 hrs beginning with the commencement of alcohol infusions for the measurement of blood alcohol concentration (BAC). A 20 µl aliquot of blood was collected into microcapillary tubes and transferred into vials that contained 0.6 N perchloric acid and 4 mM n-propyl alcohol (internal standard) in distilled water. The vials were tightly capped with a septum sealed lid and were stored at room temperature until analysis by headspace gas chromatography (Varian Associates, model 3900, Palo Alto, CA) at least 24 hr after collection. The basic gas chromatographic parameters were similar to those reported by Penton (1985), with the exception of the column (DB-wax, Megabore, J&W Scientific Folsum, CA) and the carrier gas (helium) used (West et al., 2001). Arterial blood (1 ml) was withdrawn for the measurement of partial pressures of blood gases and pH. Samples were collected anaerobically in chilled, heparinized 3 ml syringes, capped, and immediately analyzed using blood gas analyzer (ABL-5, Radiometer, Westlake, OH). The fetuses were harvested on gestational day 133. The brains were removed. The cerebellum was dissected, embedded in 4% agar, and cut sagittally into five slabs. These slabs were dehydrated through increasing concentrations of ethanol (70, 95, 100%) and then infiltrated with increasing concentrations of infiltration solution (25, 50, 75, 100% methyl methacrylate; Historesin^{TM} Embedding kit, Leica, Wetzlar, Germany). The tissue in each slab was embedded in a solution

containing 1 ml dimethyl sulfoxide (hardener) per 15 ml of 100% infiltration solution and allowed to harden. After hardening, the tissue was sectioned into 30 μm sagittal sections by using a microtome (model RM2255, Leica, Nussloch, Germany). Sections were saved, mounted on a glass slide, stained with cresyl violet, and coverslipped.

The total number of fetal cerebellar Purkinje cells was determined using unbiased stereological cell counting techniques. The Nikon (Garden City, NY) Optiphot microscope used in this study had a 40X objective lens with a 1.4 numerical aperture condenser. The microscope had a motor-driven stage to move within the x and y axes and an attached microcator to measure the z axis. The image was transferred to a personal computer (Millenium, Micron, Boise, ID) via a color video camera (model 2040, Jai, Copenhagen, Denmark). The reference volume was estimated using the Cavalieri's Principle and was calculated by the equation $V_{ref} = \Sigma p_i \text{ X } A(p_i) \text{ X } t$ where Σp_i is the total number of points (p_i) counted, $A(p_i)$ is the known area associated with each point, and t is the known distance between two serial sections counted. The GRID® software provided templates of points in various arrays that were used in point counting for reference volume estimation. The Purkinje cell density was determined by following the optical disector method, which was calculated using the formula $N_v = \Sigma Q / (\Sigma \text{disector X } A(fr) \text{ X } h)$ where ΣQ is the sum of the Purkinje cells counted from each disector frame, Σdisector is the sum of the number of disector frames counted, A(fr) is the known area associated with each disector frame, and h is the known distance between two disector planes. The placement of the disector frames was determined by the GRID® software in a random manner. The estimated total number of Purkinje cells

in the cerebellum was then calculated by multiplying the reference volume of the cerebellum and the numerical density of cells within this reference volume as described before (West et al., 2001).

Data Analysis

Two-way ANOVA with within factors of "time interval" and "day of gestation" was performed to compare BACs at different times during gestation. Fetal brain and body weights and the stereology data were analyzed using one-way ANOVA with "treatment" as the sole independent variable followed by protected Fishers LSD tests. Analyses of maternal arterial blood gases and pH were performed using a mixed ANOVA with "treatment" (only saline control and alcohol group as the normal control group was not instrumented) as the between factor and "time" as the within factor. In cases where there was a main effect, protected Fishers LSD was performed. Scheffé's test was performed to compare cell numbers of alcohol exposed fetal brains in this study and that in our third trimester study. Statistical significance was established *a priori* at $p < 0.05$.

RESULTS

A total of 8 ewes were initially assigned to each group. Animals were lost from the study as a result of fetal, but not maternal demise. The number of ewes that completed the study was similar among groups (normal control, n = 5; saline control, n = 6; alcohol, n = 5). In ewes with twin pregnancies, the fetus with the larger body weight was selected for neuronal counting (number of ewes with twins: normal control, n = 4; saline control, n = 4; alcohol, n = 2). Brains from triplets were not processed (number of ewes with triplets: saline control, n = 1). The final number of fetal brains that were processed was 5 in each of the three treatment groups (one subject in the normal control group where the cerebellum was mechanically damaged during processing was not included for statistical analysis).

Blood Alcohol Concentration (BAC)

The mean BACs measured on days 6, 40, 90 and 132 of gestation for alcohol group peaked at 1 hr (~ mg/dl) which coincided with the end of the infusion period. The blood alcohol concentrations on the different days for alcohol group did not differ significantly and were therefore combined (Figure A-6).

Fetal Brain and Body Weights

The one-way ANOVA performed on the fetal body weight data did not yield a significant main effect of treatment (normal control, 4.9 $\pm$ 0.1 kg; saline control, 4.6 $\pm$ 0.4 kg; alcohol, 5.0 $\pm$ 0.4 kg). Similarly, the ANOVAs conducted on the whole fetal

brain and cerebellum weight data showed no difference in weights among groups (whole brain: normal control, 51.6 $\pm$ 1.4 g; saline control, 50.8 $\pm$ 1.2 g; alcohol, 50.2 $\pm$ 1.3; cerebellum: normal control, 4.4 $\pm$ 0.2 g; saline control, 4.1 $\pm$ 0.2 g; alcohol, 3.9 $\pm$ 0.1 g).

Stereology Data

Estimated mean fetal cerebellar Purkinje cell numbers were significantly different among groups ($F_{2,11} = 9.15$, $p = 0.005$) (Figure A-7). Post-hoc tests revealed that the alcohol group had a significantly lower number of Purkinje cells compared with the normal control group ($p = 0.002$) and the pair-fed saline control group ($p = 0.01$). This loss in the total number of Purkinje cells was not accompanied by a change in the Purkinje cell density. However, binge alcohol exposure during all three trimesters of gestation resulted in a significantly smaller cerebellar volume reference ($F_{2,11} = 16.63$, $p < 0.001$). When comparing the cerebellar Purkinje cell numbers of alcohol exposed fetal brains in this study with that from our previous study but limiting exposure to the third trimester, we did not detect a difference (Scheffé's test; percentage loss of Purkinje cells in alcohol exposed fetuses compared with that in pair-fed saline control subjects: third trimester study, 25%; all three trimester study, 27%).

Maternal Blood Gases

The maternal arterial blood gases and pH measured from samples collected on different days of gestation were combined as they were not different. The mean $\pm$ SEM maternal arterial pH for 24 hours in response to one hour saline or alcohol infusion is

shown in Figure A-8 (top). A significant interaction was found between "time" and "treatment" ($F_{6,187} = 4.64$, $p < 0.001$). There was also a main effect of treatment ($F_{1,187} = 40.47$, $p < 0.001$) and time ($F_{6,187} = 5.27$, $p < 0.001$). Simple effect analysis indicated that the pH value at the 1st hour after the beginning of the treatment was significantly decreased in the alcohol group ($p < 0.001$) and these decreases persisted for an hour beyond the end of infusion i.e. till the 2nd hour.

The fall in pH was accompanied by a simultaneous increase in arterial partial pressure of carbon dioxide (P_aCO_2) in the alcohol group compared with that in saline control group ($F_{1,187} = 18.09$; $p < 0.001$) (Figure A-8 bottom). A main effect of time was also noted ($F_{6,187} = 3.6$; $p = 0.002$). In contrast, the maternal arterial partial pressure of oxygen (P_aO_2) was not significantly different between treatment groups or between any two time points (Table B-1).

DISCUSSION

The present findings demonstrate that binge alcohol exposure in an ovine model, where all three trimester equivalents occur *in utero*, produces a significant reduction in fetal cerebellar volume and Purkinje cell number. Even though the effects of prenatal alcohol exposure on different brain regions and cell types have been extensively studied in the rat model (Bonthius et al., 1992; Goodlett and Eilers, 1997; Hamre and West, 1993; Livy et al., 2003; Miller and Potempa, 1990; Pauli et al., 1995; West et al., 2001), this is the first stereology report on the effects of binge-like alcohol drinking throughout pregnancy on the total fetal cerebellar Purkinje cell number utilizing a model where all three trimester-equivalents of gestation occur prenatally.

When comparing Purkinje cell deficits in response to all three trimester exposure, the present findings, to those reported in our previous study utilizing the same animal model but limiting exposure to the third trimester, we did not detect a significant difference, 27% and 25% respectively. The current findings are in agreement with others who have found the third trimester-equivalent to be the most critical period of vulnerability in the rat model (Maier et al., 1999). Studies utilizing the rat model have demonstrated that alcohol administered at a dosage much higher than that used in our study (4.5 g/kg) during first two trimesters does not produce a reduction in cerebellar Purkinje cell number in rats (Maier and West, 2001b) further strengthening the conclusion that the third trimester equivalent is the period of greatest cerebellar vulnerability. One study demonstrated a greater reduction in fetal cerebellar Purkinje cell number in all three-trimester alcohol exposed rats compared with that in third

trimester exposed subjects, but failed to detect deficits when alcohol was administered during the first two trimester-equivalents (Maier et al., 1999). All these studies have lent support to the conclusion that Purkinje cells are more vulnerable to alcohol-induced depletion during differentiation than during neurogenesis (Marcussen et al., 1994). However, it is still possible that alcohol at higher doses may interfere with events that occur antecedent to Purkinje cell differentiation. The cerebellar volume deficits observed in the alcohol administered group in this study may suggest a significant loss of granule cells, the most abundant cell type in the cerebellum, in addition to the Purkinje cell loss. Prenatal alcohol exposure studies utilizing the rat model have demonstrated that losses in these two cell populations are closely correlated, likely a function of Purkinje cells being synaptic targets of granule cells (Hamre and West, 1993; Maier et al., 1999).

While much has been learned concerning critical windows of vulnerability and vulnerability of different brain regions, very little is known about the underlying mechanisms involved in alcohol mediated neuronal loss. Therefore, a secondary aim of this study was to examine the part played by arterial pH and blood gases during prenatal alcohol exposure. Our results demonstrate that the neuronal loss was accompanied by maternal normoxemia, hypercapnea and acidemia. Tissue hypoxia has been proposed as a mechanism involved in prenatal alcohol mediated injury. This hypothesis is supported by the observation that both hypoxia and fetal alcohol exposure induces similar craniofacial defects and neuronal deficits (Bronsky et al., 1986; Cudd et al., 2001b). The hippocampal CA1 pyramidal cells and the cerebellar Purkinje cells are both vulnerable

to hypoxia (Aitken and Schiff, 1986; Auer et al., 1989; Rees et al., 1999) as well as to alcohol. Studies in fetal sheep show that development of fetal brain is significantly affected by hypoxemia at mid-gestation (Rees et al., 1997). At this developmental stage, the developing cerebellum exhibits reductions in Purkinje cell number and mitosis in the external granule layer in response to hypoxemia (Rees et al., 1999). In contrast, a recent report showed that *in utero* intravenous infusion of alcohol (1.75 g/kg body weight) during the third trimester equivalent results in cerebellar Purkinje cell loss in the absence of fetal hypoxemia (Cudd et al., 2001b). Furthermore, Reynolds and colleagues (1996) found that alcohol in fact increases uterine blood flow and fetal arterial oxygen tension in near-term pregnant ewes. The findings from the present study add to the belief that the Purkinje cell loss should be attributed to mechanisms other than fetal hypoxemia. Interestingly, the present study also demonstrates a fall in maternal arterial pH following one hour infusion of alcohol. In the present study, fetuses were not instrumented and therefore fetal arterial pH and blood gases were not measured, as it is not possible to chronically instrument fetuses before mid-gestation. However, we have previously demonstrated that alcohol-mediated changes in maternal P_aCO_2 and pH_a are reflected in the fetal measurements of these variables during the third trimester (Cudd et al., 2001b). Therefore, the maternal pH_a and P_aCO_2 served as an index of these measures in the fetus in this study. Thus, the alcohol induced maternal hypercapnea in this study would directly result in fetal hypercapnea producing both intracellular and extracellular brain acidosis. Brain acidosis has been documented to cause neuronal and glial death (Goldman et al., 1989; Kraig et al., 1987; Staub et al., 1990) via multiple mechanisms

that may involve exacerbation of free radical mediated injury (Ying et al., 1999), alteration of gene expression and protein synthesis by perturbing intracellular signal transduction pathways (Siesjo et al., 1996), alteration of protein and amino acid metabolism (Milley, 1997; Safranek et al., 2003), decrease of serum IGF-1 concentrations (Brungger et al., 1997; Challa et al., 1993; Wiederkehr and Krapf, 1996), increase of glucocorticoid levels (Perez et al., 1979; Wiederkehr and Krapf, 1996; Wood and Chen, 1989). It should be noted that the threshold for acidemia or acidosis-induced death in neurons and glia is not a fixed value (Nedergaard et al., 1991). Rather, cell death is a combinatorial function of time and the degree of intracellular acidification (Nedergaard et al., 1991). Here, we propose that in addition to the duration and level of acidosis, the pattern of exposure may play a role in brain injury. To test this hypothesis, we are currently investigating if repeated periods of moderate hypercapnea and acidemia, will produce cerebellar Purkinje cell deficits, similar to that observed in response to alcohol, by manipulating the partial pressures of the maternal inspired gases.

We conclude that fetal cerebellar Purkinje cells are more sensitive to the timing of alcohol exposure and less so to the duration of exposure. The absence of decreases in maternal PaO_2 suggests that maternal hypoxia does not play a role in fetal Purkinje cell loss. And finally, we conclude that maternal arterial pH alterations mediated by alcohol exposure may play a role in alcohol-mediated damage of the developing fetal brain.

4. TEMPORAL VULNERABILITY OF PURKINJE CELLS*

INTRODUCTION

Alcohol is now broadly acknowledged to be the most prevalent human teratogen (Caetano et al., 2006; Gladstone et al., 1996). Maternal alcohol exposure can lead to a range of debilitating outcomes in the offspring that include physical, mental, behavioral and/or learning disabilities, now referred to as Fetal Alcohol Spectrum Disorder (FASD) (Riley and McGee, 2005; Sokol et al., 2003). Despite this knowledge, and after significant efforts in educating women to not drink during pregnancy, the prevalence of alcohol consumption among women of child-bearing age remains essentially unchanged (Caetano et al., 2006; CDC, 2004; Institute of Medicine, 1996; Maternal and Child Health (MCH) Data Report, 2003; NIAAA, 2000).

FASD is characterized by central nervous system (CNS) damage, at a gross, as well as microscopic level (Chen et al., 2003), and the affected children may exhibit abnormal development of the cerebellum (Archibald et al., 2001; Sowell et al., 1996; Swayze et al., 1997). The anterior cerebellar vermis is disproportionately reduced in size in the alcohol exposed subjects compared with age-matched controls (Sowell et al., 1996). Extremely small and poorly formed cerebella have been noted on autopsy in children born to alcoholic mothers (Clarren, 1977; Clarren et al., 1978; Wisniewski et

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al., 1983; Roebuck et al., 1998). An important question has been the identification of the timing of cerebellar vulnerability and whether the cerebellum is spared in women who drink early in gestation but stop by the end of the first trimester of gestation. This is a very practical question as many women who drink might stop drinking once they learn they are pregnant and are convinced that by stopping that the conceptus will be spared from damage. A number of animal studies have supported this possibility and that the cerebellum is vulnerable especially during the third trimester-equivalent. Third trimester-equivalent of human brain development alcohol exposure is considered to be especially deleterious to brain development in rats (Bonthius et al., 1992; Cragg and Phillips, 1985; Goodlett and Eilers, 1997; Goodlett et al., 1998; Hamre and West, 1993; Livy et al., 2003; Maier and West, 2001b; Maier and West, 2003; Marcussen et al., 1994; Pauli et al., 1995) and the developing cerebellar Purkinje cells are reported to be susceptible to first trimester-equivalent alcohol exposure only at exceptionally high doses (6.5 g/kg) (Maier et al., 2001b). However, human studies demonstrate that the developing cerebellum is vulnerable at all times during gestation (Peiffer et al., 1979) supporting the conclusion that even if women reduce or stop drinking after knowing that they are pregnant, injuries to the fetal cerebellum may have already occurred. Cerebellar Purkinje cell loss and disorientation have been noted in an infant whose mother binged only during the first trimester (Coulter et al., 1993). Cerebellar developmental disorders have also been identified in children by MRI, who had been exposed to alcohol prenatally during first, first and second, or all three trimesters of gestation (Autti-Ramo et al., 2002).

Although no quantitative data regarding neuronal loss are available from human FAS studies, data from rat studies have shown that alcohol exposure causes a significant reduction in cerebellar Purkinje cell number but only if the alcohol administration period includes the period of brain growth spurt like that during the third trimester in humans. The maximum velocity of brain growth occurs at the time of parturition in humans, whereas it occurs postnatally in rats (Dobbing and Sands, 1973; Dobbing and Sands, 1979), requiring the assessment of the response to alcohol administration during the third trimester-equivalent of human brain development in rats to be conducted postnatally. Therefore, to extrapolate the findings from rats to humans, one must assume that the intrauterine environment, placenta, mother and parturition play a limited role in mediating the damage (Cudd, 2005). We conjectured that damage from prenatal alcohol exposure may involve the intrauterine environment and maternal interactions and

therefore chose the sheep model, where the entire gestational equivalent of human brain development occurs *in utero*, to address the question of temporal vulnerability of the cerebellum. We hypothesized that maternal alcohol binging during the first or third trimester-equivalents would result in damage of the cerebellar Purkinje cells. We also wished to compare the fetal cerebellar Purkinje cell number with those from an earlier study (Ramadoss et al., 2007b), where the sheep received alcohol *in utero* throughout gestation. In all studies, a weekend binge pattern was employed, a drinking pattern common in women who use alcohol during pregnancy (Caetano et al., 2006; Cudd et al., 2001a; Ebrahim et al., 1999; Gladstone et al., 1996; Maier and West, 2001a). A dose of 1.75 g/kg of alcohol was used to achieve blood alcohol concentrations (BACs) between 200 and 300 mg/dl; values that have been reported for mothers of children with FAS (Church and Gerkin, 1988) and in women who abuse alcohol (Urso et al., 1981).

METHODS

Animals and Breeding

The experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University. Suffolk ewes (aged 2 to 6 years) were bred under controlled conditions as described elsewhere (Ramadoss et al., 2006a). The day of mating (the day that ewes were marked by the ram) was designated as gestational day (GD) 0. Ewes were then maintained in an environmentally regulated facility (22ºC and a 12:12 light/dark cycle) where they remained for the duration of the experiments. Animals in all treatment groups were fed 2 kg/day of a "complete" ration (Sheep and Goat Pellet, Producers Cooperative, Bryan, TX) and they consumed all of the feed offered.

Experimental Protocol

Treatment groups

There were four treatment groups (n = 5 for each treatment group) in this study: first trimester-equivalent pair-fed saline control group, first trimester-equivalent alcohol group (1.75 g/kg), third trimester-equivalent pair-fed saline control group, and third trimester-equivalent alcohol group (1.75 g/kg).

First trimester-equivalent groups

In the first trimester-equivalent groups, on gestational day 4, the beginning of the first trimester-equivalent in this species, an intravenous catheter (16 ga., 5.25 inch Angiocath™, Becton Dickinson, Sandy, UT) was placed into the jugular vein of the

subjects. Alcohol or saline was administered intravenously (IV) over a one-hour period via a peristaltic pump (Masterflex, Model 7014-20, Cole-Parmer, Niles, IL) in a binge-like paradigm (modeling weekend only binge drinking), consisting of three consecutive days of exposure followed by four days without exposure and this pattern was repeated beginning on gestational day (GD) 4 until GD 42 (Figure A-9). Subjects were sacrificed on day 133 (term is 147 days) for fetal brain processing. The alcohol solution was prepared by adding 95% alcohol to sterile 0.9% saline to achieve a 40% w/v alcohol solution. Solutions were prepared under aseptic conditions and were administered through a 0.2 μm bacteriostatic filter. The pair-fed saline control group (SC) received an infusion of isotonic saline (0.9%) that was equal in volume to the 1.75 g/kg alcohol infusion. Pumps were calibrated before each infusion.

Third trimester-equivalent groups

In the third trimester-equivalent groups, on gestational day 102, the ewes underwent surgery to chronically implant femoral arterial and venous polyvinyl chloride catheters (0.050" inner diameter, 0.090" outer diameter) as previously described (Cudd et al., 2001b). In brief, anesthesia was induced by administering diazepam (0.2 mg/kg intravenously, Abbott Laboratories, North Chicago, IL) and ketamine (4 mg/kg intravenously, Ketaset®, Fort Dodge, IA). The ewes were intubated and a surgical plane of anesthesia was maintained using isoflurane (0.5-2.5%, IsoFlo®, Abbott Laboratories, North Chicago, IL) and oxygen. Arterial and venous catheters were advanced into the aorta and vena cava via the femoral artery and vein respectively. At the end of surgery, the ewes received an injection of flunixin meglumine (1.1 mg/kg intramuscularly,

Banamine®, Scherring-Plough, Union, NJ), a prostaglandin synthase inhibitor, to reduce postoperative pain. Ewes also received postoperative antibiotics (ampicillin trihydrate, polyflex®, Aveco, Fort Dodge, IA, 25 mg/kg administered subcutaneously for 5 days and gentamicin sulfate, Gentavet®, Velco, St.Louis, 2 mg/kg administered intramuscularly twice daily for 5 days). Alcohol or saline was then administered intravenously (IV) over a one-hour period similar to the first trimester-equivalent groups in a binge-like paradigm, beginning on GD 109 till 132. Subjects were sacrificed on day 133.

Blood alcohol concentration (BAC) measurement

Blood was drawn for the measurement of BAC from the jugular vein catheter in the first trimester-equivalent groups and from the femoral artery catheter from the third trimester-equivalent groups at both the commencement and the end of alcohol infusion (one hour), when the BAC is known to reach its peak level (Cudd et al., 2001b; West et al., 2001; Ramadoss et al., 2007b). A 20 µl aliquot of blood was collected into microcapillary tubes and transferred into vials that contained 0.6 N perchloric acid and 4 mM n-propyl alcohol (internal standard) in distilled water. The vials were tightly capped with a septum sealed lid and were stored at room temperature until analysis by headspace gas chromatography (Varian Associates, model 3900, Palo Alto, CA) at least 24 hr after collection. The basic gas chromatographic parameters were similar to those reported by Penton (1985), with the exception of the column (DB-wax, Megabore, J&W Scientific Folsum, CA) and the carrier gas (helium) used (West et al., 2001).

Fetal Cerebellar Tissue Processing

On GD 133, the ewes were euthanized using sodium pentobarbital (75 mg/kg, intravenously), and the fetuses were removed from the uterus and perfused with saline followed by cold fixative solution containing 1.25% paraformaldehyde and 3% glutaraldehyde in phosphate buffer (pH, 7.4). The brains were removed and stored in additional fixative until processed for stereological cell counting.

The cerebellum was dissected, embedded in 4% agar, and cut sagittally into five slabs. These slabs were dehydrated through increasing concentrations of ethanol (70, 95, 100%) and then infiltrated with increasing concentrations of infiltration solution (25, 50, 75, 100% methyl methacrylate; Historesin^{TM} Embedding kit, Leica, Wetzlar, Germany). The tissue in each slab was embedded in a solution containing 1 ml dimethyl sulfoxide (hardener) per 15 ml of 100% infiltration solution and allowed to harden. After hardening, the tissue was sectioned into 30 μm sagittal sections by using a microtome (model RM2255, Leica, Nussloch, Germany). Every twentieth section was saved, mounted on a glass slide, stained with cresyl violet, and coverslipped.

Stereological Cell Counting

The total number of fetal cerebellar Purkinje cells was estimated using unbiased stereological cell counting techniques as described previously (Ramadoss et al., 2007b). In brief, the Nikon (Garden City, NY) Optiphot microscope used in this study had a 40X objective lens with a 1.4 numerical aperture condenser. The microscope had a motor-driven stage to move within the x and y axes and an attached microcator to measure the z

axis. The image was transferred to a personal computer (Millenium, Micron, Boise, ID) via a color video camera (model 2040, Jai, Copenhagen, Denmark). The reference volume was estimated using the Cavalieri's Principle and was calculated by the equation $V_{ref} = \Sigma p_i \text{ X } A(p_i) \text{ X } t$ where Σp_i is the total number of points (p_i) counted, $A(p_i)$ is the known area associated with each point, and t is the known distance between two serial sections counted. The GRID® software provided templates of points in various arrays that were used in point counting for reference volume estimation. The Purkinje cell density was determined by following the optical disector method, which was calculated using the formula $N_v = \Sigma Q / (\Sigma \text{disector X } A(fr) \text{ X } h)$ where ΣQ is the sum of the Purkinje cells counted from each disector frame, Σdisector is the sum of the number of disector frames counted, A(fr) is the known area associated with each disector frame, and h is the known distance between two disector planes. The placement of the disector frames was determined by the GRID® software in a random manner. The estimated total number of Purkinje cells in the cerebellum was then calculated by multiplying the reference volume

of the cerebellum and the numerical density of cells within this reference volume as described before (West et al., 2001).

Data Analysis

Fetal growth parameters and the stereology data were analyzed using a two-way ANOVA with ”treatment” (saline or alcohol) and “exposure period” (first or third trimester-equivalent) as between factors. Simple effect analysis was performed should there be an interaction between “treatment” and “exposure period”. Scheffé’s test was performed to identify differences in the estimated total cerebellar Purkinje cell numbers between the first and third trimester-equivalent alcohol exposed fetal brains in this study with that in our all three trimester-equivalent study. Statistical significance was established *a priori* at $p < 0.05$ while p values between 0.05 and 0.10 were considered trends.

RESULTS

Blood Alcohol Concentration (BAC)

The mean ± SEM maternal BACs at the end of alcohol infusion (1 hr; the time point when BACs were known to peak) (Cudd et al., 2001b; West et al., 2001; Ramadoss et al., 2007b), were 206 ± 12 mg/dl and 260 ± 10 mg/dl in the first and third trimester-equivalent alcohol exposed subjects respectively. The subjects remained conscious throughout and after the alcohol infusion but appeared ataxic if encouraged to walk shortly after the end of the infusion.

Fetal Growth Measurements

A two-way ANOVA performed on the fetal body weight and length data did not identify a significant main effect of treatment, exposure period, or an interaction between the two factors (weight: first trimester-equivalent saline control, 5.1 ± 0.4 kg; first trimester-equivalent alcohol, 4.8 ± 0.4 kg, third trimester-equivalent saline control, 4.7 ± 0.3 kg; third trimester-equivalent alcohol, 4.9 ± 0.2 kg; length: first trimester-equivalent saline control, 49.7 ± 1.3 cm; first trimester-equivalent alcohol, 49.5 ± 1.6 cm; third trimester-equivalent saline control, 54.4 ± 0.9 cm; third trimester-equivalent alcohol ,52.0 ± 0.7 cm).

Stereology Data

A two-way ANOVA conducted on the total fetal cerebellar Purkinje cell number revealed a main effect of treatment ($F_{1,16} = 37.92$, $p < 0.001$) (Figure A-10). However,

there was no significant main effect of the exposure period ($F_{1,16} = 0.49$, $p = 0.495$) or an interaction between the two factors ($F_{1,16} = 1.49$, $p = 0.25$). The alcohol treated subjects had a significantly lower number of Purkinje cells compared with those in the pair-fed saline control group ($p < 0.001$) regardless of the period of exposure. The loss in the total number of Purkinje cells was also accompanied by a significant change in the cerebellar Purkinje cell density (Figure A-11). The two-way ANOVA analysis conducted on the cell density data revealed a significant main effect of treatment ($F_{1,16} = 20.37$, $p < 0.001$), but did not yield a main effect of the exposure period ($F_{1,16} = 0.12$, $p = 0.734$) or an interaction between the two factors ($F_{1,16} = 2.52$, $p < 0.14$). The alcohol exposed fetuses had a significantly lower cell density compared with the controls ($p < 0.001$) irrespective of the period of exposure. With regard to the cerebellar volume reference, an interaction between the exposure period and treatment was observed ($F_{1,16} = 9.16$, $p = 0.008$) (Figure A-12). A significant main effect of treatment ($F_{1,16} = 4.72$, p

= 0.045), but not the period of administration ($F_{1,16}$ = 0.007, p = 0.935) was noted. Simple effect analysis showed that third trimester-equivalent alcohol treated subjects had a smaller cerebellar volume reference compared with that in the third trimester-equivalent saline control group. However, such differences were not found while comparing between the first trimester-equivalent pair-fed saline control and first trimester-equivalent alcohol exposed subjects. In general, no differences in any of the stereology measures were found between the first trimester-equivalent and third trimester-equivalent saline control subjects. When comparing the present findings to those from our previous study but extending the duration of alcohol exposure to all three trimester-equivalents of gestation, we did not detect a difference in fetal cerebellar Purkinje cell number (Scheffé's test; total cell number: first trimester-equivalent alcohol exposed subjects, 4.9 x 10^6; third trimester-equivalent alcohol exposed subjects, 4.6 x 10^6; all three trimester-equivalent alcohol exposed subjects, 4.8 x 10^6).

DISCUSSION

The Cerebellum Is an Important Target in FASD

Consistent with previous FASD literature, this study demonstrated a significant reduction in cerebellar Purkinje cell number in the alcohol exposed fetuses compared with those in the controls. Cerebellar anomalies have been a common finding in human as well as animal studies in response to developmental alcohol exposure. Clinically, one of the most common abnormalities found in human prenatal alcohol exposure pathology studies has been the dysgenesis of cerebellum and/or brainstem (Clarren et al., 1978; Peiffer et al., 1979; Wisniewski et al., 1983). A significantly smaller anterior cerebellar vermis has been noted in children who have been exposed to large amounts of alcohol (Sowell et al., 1996). Autti-Ramo and colleagues (2002) suggest that the cerebellum is the most sensitive morphological indicator of prenatal alcohol exposure in children. A positron emission spectroscopy study by Hannigan and coworkers (1995), similarly demonstrated that three of the four alcohol exposed children exhibited lower levels of glucose metabolism in the cerebellum than control subjects. Complementing these human studies, quantitative stereology studies conducted utilizing animal models, especially the rat, further support this teratogenic effect of alcohol on the developing cerebellum. Findings from West and his research group have demonstrated the cerebellum to be more vulnerable to developmental alcohol exposure than any other brain structure and the cerebellar Purkinje cells to be more susceptible than any other cell type (Bonthius and West, 1991; Maier et al., 1997; Maier and West, 2001b).

First Trimester-Equivalent Alcohol Exposure Reduces Fetal Cerebellar Purkinje Cell Number

A significant finding from this study is that fetal cerebellar Purkinje cell loss occurs in response to alcohol exposure limited to the first trimester-equivalent, a finding that strongly supports a number of human studies. Peiffer and coworkers (1979) first suggested that the teratogenic effects of alcohol may involve a broad period of exposure and that drinking during the first trimester of gestation can cause significant CNS damage. Later, Coulter et al., 1993 reported significant cerebellar Purkinje cell loss accompanied by misalignment of Purkinje cells and abnormal dendritic structure from an autopsy case of a 2.5 month-old girl whose mother's drinking was limited to the first trimester of pregnancy. Cerebellar hypoplasia was identified by MRI in school children who had been exposed to alcohol prenatally during the first trimester only, and these cerebellar deficits were closely correlated with poor results on the repetition of Nonsense Words subtest and Visual Attention subtest (Autti-Ramo et al., 2002). Even though a broad period of vulnerability has similarly been suggested by animal studies utilizing the rat model (West and Ward, 1992), the reduction in neurons has been shown to depend heavily upon the timing of alcohol exposure relative to different phases of brain development (Bonthius et al., 1992; Cragg and Phillips, 1985; Goodlett and Eilers, 1997; Goodlett et al., 1998; Hamre and West, 1993; Livy et al., 2003). The developing cerebellar Purkinje cells in rats are considered to be most vulnerable during the third trimester-equivalent (Maier et al., 1999) and are susceptible to first trimester-equivalent alcohol exposure only at exceptionally high doses (6.5 g/kg) (Maier et al., 2001b)

suggesting that the Purkinje cells are more vulnerable to alcohol-induced depletion during differentiation than during neurogenesis (Marcussen et al., 1994) and that the rat first trimester-equivalent is vulnerable, albeit only at high alcohol doses. Further, when alcohol was administered during all three trimester-equivalents of gestation, the cell numbers were significantly lower compared with that when the period of exposure was restricted to the third trimester-equivalent, even though the alcohol exposure during the first or second trimester-equivalent only did not result in a significant cell loss per se (Maier et al., 1999). Thus, studies utilizing the rat model differ somewhat from the present study and from clinical reports with respect to the identification of a most sensitive period of development. We speculate that this difference may be due to at least two reasons: 1) all three trimester-equivalents are *in utero* in sheep, as in humans, but the third trimester-equivalent is a postnatal event in rats, 2) there are differences in the ontogeny of cerebellar Purkinje cell among species.

Maternal-Fetal Interaction Plays a Role in Alcohol Mediated Fetal Cerebellar Damage

The maximum velocity of brain growth occurs at parturition in humans, whereas it occurs postnatally in rats (Cudd, 2005; Dobbing and Sands, 1973; Dobbing and Sands, 1979). Therefore, the use of the rat third trimester-equivalent model involves postnatal alcohol exposure which requires the assumption that the intrauterine environment, placenta, maternal interactions and parturition do not play an important role in mediating the damage (Cudd, 2005). Third trimester-equivalent binge exposure produces similar

Purkinje cell losses in rats and sheep, but at lower peak BACs in the sheep (peak BAC: sheep, 260 mg/dl; rat, 374 mg/dl, Goodlett and Eilers, 1997). While one explanation for this difference when comparing between species is that rat Purkinje cells are inherently less sensitive to alcohol, we have provided evidence that intrauterine mechanisms and maternal-fetal interactions play a role in mediating fetal cerebellar Purkinje cell loss (Cudd et al., 2001b; Ramadoss et al., 2007a; West et al., 2001). Binge drinking alters maternal ventilation, acid-base balance, cardiovascular homeostasis, uterine blood flow, and endocrine function during gestation, actions that result in changes in the fetus that may play an important role in prenatal ethanol mediated injury (Cudd et al., 2001a; Cudd et al., 2001b; Ramadoss et al., 2007b; Ramadoss et al., 2006b; Reynolds et al., 1996). While these actions could explain the species differences in sensitivity to third trimester-equivalent exposure, it does not explain the species differences in response to first trimester-equivalent exposure.

A Note on the Ontogeny of the Ovine Cerebellar Purkinje Cell

In order to reasonably extrapolate findings from animal studies to humans, it is important to consider the differences in the ontogeny of cerebellar Purkinje cells when comparing among species. The development of Purkinje cells in sheep is much more advanced than that in humans as well as in monkeys, just prior to birth, and remarkably more developed than that in the rat (Rees and Harding, 1988). The cerebellar Purkinje cells begin to differentiate rapidly between postnatal day 4 and 20 in the rat. In humans, Purkinje cell differentiation begins in fetal month 4 (Jacobson, 1991), and by parturition

have completed the second stage of maturation consisting of arrangement of Purkinje cells in monolayer, formation of dendritic processes and formation of spines in secondary and tertiary dendrites (Rees and Harding, 1988). In sheep, the development of the Purkinje cell is even more advanced than in humans; they in fact acquire the adult form by day 140 of gestation (Rees and Harding, 1988). The birth date of the cerebellar Purkinje cell in sheep is not well documented. However, it is speculated that the birth date of the cerebellar Purkinje cell in this species is similar to that in calves (proportionate to the length of gestation); the relatively advanced degree of motor coordination demonstrated by both species at birth likely closely correlates with the state of maturation of Purkinje cells. The formation of Purkinje cells is completed by about day 100 of gestation in calves (parturition: GD, 285), and they continue to differentiate in conjunction with the development of the cerebellum (de Lahunta, 1983). However, based on the current findings and the data from human studies, it still cannot be ruled out that alcohol may interfere with events that occur antecedent to Purkinje cell differentiation. Alcohol may alter cell cycle kinetics during Purkinje cell neurogenesis, as well as cell migration and very early differentiation (Marcussen et al., 1994). Therefore, it is possible that the mechanisms responsible for alcohol mediated Purkinje cell loss may be different in late and early exposures, differentially affecting the differentiating neurons and the neuronal precursor cells respectively. However, clearly further investigation of the ontogeny of the ovine fetal Purkinje cells is warranted in order to better understand the mechanisms behind first and third trimester-equivalent damage.

Cerebellar Purkinje Cells Are Not Completely Depleted

A nearly identical magnitude of cell loss was seen in alcohol group subjects regardless of the time of exposure; i.e. the number of Purkinje cells lost in the all three trimester-equivalent alcohol exposed subjects was not the sum of the numbers lost during the first and the third trimester-equivalent: rather it was a similar number. This finding is in agreement with a number of studies conducted utilizing the rat model where the cerebellar Purkinje cells were never completely depleted irrespective of the dosage or the duration of alcohol exposure (Goodlett and Eilers, 1997; Goodlett et al., 1998; Maier et al., 1999; Maier and West, 2001b). Studies conducted using the rat model have suggested that within the cerebellum, there is a difference in vulnerability; lobules I, IX and X are more vulnerable than lobules VI and VII (Bonthius and West, 1990; Maier et al., 1997). Thus, this would suggest that irrespective of the duration or level of alcohol administered, a certain number of Purkinje cells would remain. Alternately, it can be stated that only a certain population of Purkinje cells are susceptible to alcohol induced depletion, supporting our findings that alcohol exposure at any time during pregnancy results in loss of a fixed number of cells. Future studies will investigate lobule-specific differences in Purkinje cell loss in the first, third, and all three trimester-equivalent alcohol exposed ovine fetuses.

SUMMARY

This is the first animal study to report a loss in fetal cerebellar Purkinje cells, irrespective of the period of alcohol consumption, in a model where all three trimester-equivalents of gestation occur *in utero*, as occurs in humans. These findings suggest that there is no safe period for drinking. Thus, women who engage in binge drinking during the first trimester are at a high risk of giving birth to children with cerebellar damage even if drinking ceases after the first trimester. Finally, these results support the hypothesis that not all cerebellar Purkinje cells are depleted irrespective of the duration of drinking, and only a certain population of cells are vulnerable to alcohol induced depletion.

5. THREE TRIMESTER ALCOHOL EFFECTS ON THE HIPPOCAMPUS

INTRODUCTION

Maternal alcohol exposure can lead to debilitating outcomes in the offspring that range from severe physical and mental deficits to milder behavioral and learning disabilities, now referred to as Fetal Alcohol Spectrum Disorders (FASD) (Riley and McGee, 2005; Sokol et al., 2003). Serious behavioral problems and altered performance in learning and/or memory tasks in FASD children have been reported (for review, see Mattson et al., 2001), and these deficits have been closely related to hippocampal injury. For instance, it has been documented that prenatally alcohol exposed children exhibit deficits in spatial memory as well as delayed object recall (Uecker and Nadel, 1996), impairments thought to be closely associated with hippocampal function. Another study conducted in FASD children reported deficits in place learning and spared cue-navigation, suggesting hippocampal damage (Hamilton et al., 2003). These behavioral studies are further supported by MRI studies where FASD children have been shown to display volume asymmetries in the hippocampus, with the volume of the left hippocampus being smaller than its counterpart in the right lobe (Riikonen et al., 1999).

Although there is no quantitative study in the human literature regarding altered number of hippocampal neurons that could explain the behavioral deficits in prenatally alcohol exposed children, a number of animal studies utilizing the rat model have reported differences in the number of neurons in alcohol exposed subjects compared with that in the controls. West and coworkers (1986) reported a 10% increase in the

granule cell number in response to third trimester-equivalent alcohol exposure. Miller (1995) demonstrated an increase in dentate gyrus granule cell number in postnatal rat pups in response to moderate alcohol exposure and a decrease in their number in response to higher blood alcohol concentrations (BACs). Further, they also observed decreased CA1 pyramidal cell number in response to moderate doses of prenatal alcohol exposure, and no decreases to these same BACs when exposed postnatally. Another recent study by Livy and coworkers demonstrated decreased number of granule cells, CA1, and CA3 pyramidal cells in response to high doses of alcohol (BAC, 339 mg/dl), but only when the exposure period included the third trimester-equivalent (Livy et al., 2003). Similar to the results of Livy et al., Maier and West (2001b), demonstrated no reductions in hippocampal cell numbers when the alcohol administration period included only the first two trimester-equivalents. One reason for these differences among studies is the methodology used to estimate the number of cells in the hippocampal formation. Some of the above studies used only density measures, and others used stereological techniques with or without optical disector procedure. The other reasons could be differences in the exposure paradigm (chronic as against binge), the mode of administration, and alcohol dosage.

It should be noted that the rat third trimester-equivalent occurs postnatally. The maximum velocity of brain growth occurs at the time of parturition in humans, whereas it occurs postnatally in rats (Dobbing and Sands, 1973; Dobbing and Sands, 1979), requiring the assessment of the response to alcohol administration during the third trimester-equivalent of human brain development in rats to be conducted postnatally.

Therefore, to extrapolate the findings from rats to humans, one must assume that the intrauterine environment, placenta, mother and parturition play a limited role in mediating the damage (Cudd, 2005; Ramadoss et al., 2007a). We conjectured that damage from prenatal alcohol exposure may involve the intrauterine environment and maternal interactions and therefore chose the sheep model, where the entire gestational equivalent of human brain development occurs *in utero*, to address the question of hippocampal vulnerability in response to alcohol exposure throughout gestation. We hypothesized that maternal alcohol binging during all three trimester-equivalents would result in reduced cell numbers in the hippocampal formation. We specifically examined the effect on the pyramidal cells of the CA1-3 field, and the granule cells of the dentate gyrus. We employed a weekend binge pattern, a drinking pattern common in women who use alcohol during pregnancy (Caetano et al., 2006; Ebrahim et al., 1999; Gladstone et al., 1996; Maier and West, 2001a; Ramadoss et al., 2007a) and who are more likely to refrain compared to everyday drinkers, if convinced that drinking poses a hazard to their baby.

METHODS

Animals and Breeding

The experimental procedures employed in this study were approved by the University Laboratory Animal Care Committee at Texas A&M University. Suffolk ewes (aged 2 to 6 years) maintained on coastal Bermuda grass pasture and supplemented with alfalfa hay were bred under controlled conditions. Time dated pregnancies were achieved by controlling the estrous cycle through the use of progesterone impregnated vaginal implants (EAZI-BREED™, CIDR®, Pharmacia & Upjohn Ltd., Auckland New Zealand). Implants were removed 11 days after placement at which time prostaglandin $F_{2\alpha}$ (LUTALYSE®, Pharmacia & Upjohn, Kalamazoo MI, 20 mg) was intramuscularly administered. The following day, ewes were placed with a ram fitted with a marking harness for a period of 24 hours. Marked ewes were assessed ultrasonographically on 25, 60 and 90 days to confirm pregnancy. On day 4 of gestation, the saline control and alcohol group subjects were moved into individual pens but were able to see herdmates in the adjacent pens at all times. Conditions of constant temperature (22 ^{0}C) and fixed light dark cycle (12:12) were maintained. Once confined, the saline and alcohol treatment group subjects received 2 kg/day of a complete ration (Sheep and Goat Pellet, Producers Cooperative Association, Bryan, TX). Daily feed consumption was monitored; subjects in the alcohol and saline treatment groups consumed all of the food offered.

Alcohol Dosing Protocol

Two treatment groups, an alcohol group (alcohol dosage of 1.75 g/kg body weight), and a saline control group that received 0.9% saline of a volume of and at an infusion rate equivalent to that of the alcohol dose were studied. Infusion solutions were delivered intravenously by peristaltic pump (Masterflex, model 7014-20, Cole parmer, Niles IL) through a 0.2 µm bacteriostatic filter. Pumps were calibrated before infusion. Alcohol infusions were 40% w/v in sterile saline administered over one hour. Alcohol was administered on three consecutive days followed by 4 days without alcohol beginning on day 4 of gestation.

Experiment Protocol

Details of the experiment protocol have been described earlier (Ramadoss et al., 2006a). In brief, our experiments began on day 4 and were terminated on day 133 of gestation. On gestational day 4, an intravenous catheter (16 gauge, 5.25 in Angiocath™ Becton Dickinson, Sandy, UT) was placed percutaneously into the jugular vein. On the day of infusions, ewes were connected to the infusion pump by 0830 hr and alcohol was infused continuously over 1 hr, between 0830 and 0930 hr. On gestational day 42, after conception and pregnancy was confirmed ultrasonographically, the ewes underwent surgery to chronically implant femoral arterial and venous vascular access ports (V-A-P™, Model CP 47P, Access Technologies, Skokie IL). The ewes were not surgically instrumented until after the first trimester to avoid early embryonic loses. Infusions were then given through the venous port and blood was sampled from the arterial port.

Blood Alcohol Concentration (BAC) Measurement

Blood was drawn from the jugular vein catheter on gestational days 6 and 40 and from femoral artery catheter on days 90 and 132 every 30 min for 2 hr and at 6 and 24 hrs beginning with the commencement of alcohol infusions for the measurement of BAC. A 20 μl aliquot of blood was collected into microcapillary tubules and transferred into vials that contained 0.6 N perchloric acid and 4 mM n-propyl alcohol (internal standard) in distilled water. The vials were tightly capped with a septum sealed lid and were stored at room temperature until analysis by headspace gas chromatography (Varian Associates model 3400, Palo Alto, CA) at least 24 hr after collection. The basic gas chromatographic parameters were similar to those reported by Penton (1985), with the exception of the column (DB-wax, Megabore, J&W Scientific Folsum, CA) and the carrier gas (helium) used (West et al., 2001).

Fetal Hippocampal Tissue Processing

On GD 133, the ewes were euthanized using sodium pentobarbital (75 mg/kg, intravenously), and the fetuses were removed from the uterus and perfused with saline followed by cold fixative solution containing 1.25% paraformaldehyde and 3% glutaraldehyde in phosphate buffer (pH, 7.4). The brains were removed and stored in additional fixative until processed for stereological cell counting.

The fetal brain was divided parasagittally and the right hippocampus was removed. The hippocampal tissue was then dehydrated through increasing concentrations of alcohol (70, 95, 100%) and then infiltrated with increasing

concentrations of infiltration solution (25, 50, 75, 100% methyl methacrylate; Historesin^{TM} Embedding kit, Leica, Wetzlar, Germany). Following infiltration, the tissue was embedded in a solution containing 1 ml dimethyl sulfoxide (hardener) per 15 ml of 100% infiltration solution and allowed to harden. After hardening, the tissue was sectioned coronally into 30 μm sections by using a microtome (model RM2255, Leica, Nussloch, Germany). Sections were mounted serially on glass slides, stained with cresyl violet, and coverslipped.

Stereological Cell Counting

The total number of fetal hippocampal pyramidal cells in the CA1, CA2/3 fields, and the granule cells in the dentate gyrus were determined using unbiased stereological cell counting techniques as described before (Ramadoss et al., 2007b; West et al., 2001). The term CA2/3 refers to the combined measure of the CA2 and CA3 fields due to the difficulty in differentiating these two fields accurately by stereological procedures. The Nikon (Garden City, NY) Optiphot microscope used in this study had a 4X objective lens for volume measures and a 60X objective lens with a 1.4 numerical aperture condenser for density measures. The microscope had a motor-driven stage to move within the x and y axes and an attached microcator to measure the z axis. The image was transferred to a personal computer (Millenium, Micron, Boise, ID) via a color video camera (model 2040, Jai, Copenhagen, Denmark). The reference volume was estimated using the Cavalieri's Principle and was calculated by the equation $V_{ref} = \Sigma p_i \text{ X } A(p_i) \text{ X } t$ where Σp_i is the total number of points (p_i) counted, $A(p_i)$ is the known area associated

with each point, and t is the known distance between two serial sections counted. The GRID® software provided templates of points in various arrays that were used in point counting for reference volume estimation. The cell density was determined by following the optical disector method, which was calculated using the formula $N_v = \Sigma Q / (\Sigma disector \times A(fr) \times h)$ where ΣQ is the sum of the hippocampal cells counted from each disector frame, Σdisector is the sum of the number of disector frames counted, A(fr) is the known area associated with each disector frame, and h is the known distance between two disector planes. The placement of the disector frames was determined by the GRID® software in a random manner. The estimated total number of cells in the dentate gyrus, CA1, and the CA2/3 fields were then calculated by multiplying the reference volume of the respective regions and the numerical density of cells within this reference volume as described before (West et al., 2001).

Data Analysis

Data are presented as mean ± SEM. All stereology measures were analyzed using a one-way ANOVA with treatment group as 'between' factor. Statistical significance was established *a priori* at $p < 0.05$.

RESULTS

A total of 8 ewes were initially assigned to each group. Animals were lost from the study as a result of fetal, but not maternal demise. The number of ewes that completed the study was similar among groups (saline control, n = 6; alcohol, n = 5). In ewes with twin pregnancies, the fetus with the larger body weight was selected for neuronal counting (number of ewes with twins: saline control, n = 4; alcohol, n = 2). Brains from triplets were not processed (number of ewes with triplets: saline control, n = 1). The final number of fetal brains that were processed was 5 in each of the groups.

Blood Alcohol Concentration

The mean BACs measured on days 6, 40, 90 and 132 of gestation for alcohol group peaked at 1 hour which coincided with the end of infusion. The mean peak BAC was 189 $\pm$ 19 mg/dl.

Stereology Data

The estimated mean total number of fetal dentate gyrus granule cells was not different among groups ($F_{1,8} = 0.113$, $p = 0.746$) in response to maternal alcohol binging

throughout gestation (Figure A-13). The volume of the dentate gyrus was also not altered ($F_{1,8}$ = 2.349, p = 0.164). However, all three trimester-equivalent fetal alcohol exposure resulted in a significant increase (~20%) in the density of the granule cells compared with that in the pair-fed saline control group ($F_{1,8}$ = 7.295, p = 0.027).

The estimated mean total number of fetal hippocampal pyramidal cells in the CA2/3 field was also not different among groups ($F_{1,8}$ = 0.044, p = 0.839) (Figure A-14). Further, neither the volume ($F_{1,8}$ = 0.015, p = 0.906) of the CA2/3 pyramidal field nor the cell density ($F_{1,8}$ = 0.174, p = 0.688) were decreased in response to alcohol.

The estimated mean total pyramidal cell number in the CA1 field was also not different in the alcohol group compared with that in the pair-fed saline control group ($F_{1,8}$ = 1.346, p = 0.279) (Figure A-15). Similar to the results of the CA2/3 field, the volume reference of the CA1 field ($F_{1,8}$ = 0.570, p = 0.472) and the cell density ($F_{1,8}$ = 0.236, p = 0.640) were not different between the groups.

DISCUSSION

The Effects of Three Trimester-Equivalent Alcohol Exposure on the Ovine Fetal Hippocampal Formation

To our knowledge, this is the first report on the effects of binge-like alcohol drinking throughout pregnancy on the fetal hippocampal cell numbers utilizing stereology and a model where all three trimester-equivalents of gestation occur prenatally. Here, we report a selective increase in the density of granule cells in the dentate gyrus, although the total number of cells was not altered. Further, the pyramidal cell number in the CA2/3 field and the CA1 field were not decreased in response to all three trimester-equivalent alcohol exposure in fetal sheep. This is a very important finding as FASD children are associated with behavioral deficits, that include altered performance in tasks such as learning and memory (Mattson et al., 2001). Children exposed to alcohol prenatally exhibit hippocampus-associated impaired learning (Hamilton et al., 2003), and memory deficits (Uecker and Nadel, 1996), and these impairments have been correlated to hippocampal structural deficits (Riikonen et al., 1999). Although no quantitative data regarding neuronal loss are available from human FAS studies, data from rat studies have shown that developmental alcohol exposure causes alterations in neuronal numbers of different cell types within the hippocampal formation and these alterations are shown to be dependent on the period of alcohol exposure. However, it should be noted that the third trimester-equivalent in rats occurs postnatally, and that any extrapolation from the cell count studies conducted utilizing the rat model to humans would require the assumption that maternal-fetal interactions,

placenta and parturition play no role in fetal neuronal damage. Therefore, we performed this study to examine the effects of alcohol on fetal hippocampal cell types in sheep, where the entire gestational-equivalent of human brain development occurs *in utero*.

The Effect of Three Trimester-Equivalent Binge Alcohol Exposure on the Fetal Dentate Gyrus Granule Cells

Similar to our results, several studies utilizing the rat model have demonstrated altered number and/or density of dentate gyrus granule cells following developmental alcohol exposure. West and coworkers (1986) reported a 10% increase in the granule cell number in response to third trimester-equivalent alcohol exposure (BAC, 380 mg/dl). However, this study did not use stereological cell counting techniques and the result was based on density measures. Miller (1995) used stereological techniques but not the optical disector method to demonstrate an increase in dentate gyrus granule cell number in postnatal rat pups in response to moderate alcohol exposure (BAC, 132 mg/dl), and a decrease in their number in response to BACs of 339 mg/dl. Further, first two trimester-equivalent alcohol exposure did not alter the cell number. Similarly, Livy and coworkers (2003), using stereological optical disector method, showed decreased granule cell numbers in response to a high BAC of 339 mg/dl, but only when the exposure period included the third trimester-equivalent. Maier and West (2001b), again using stereological optical disector technique, showed that none of the hippocampal cell types were altered in response to alcohol exposure during the first two trimester-equivalents. Consistent with these findings in the rat model, we have shown that a BAC

of 189 mg/dl increased the packing density of granule cells in fetal sheep, but the total number was not altered. Thus, we and others have shown an increase in density and/or number in response to moderate alcohol exposure and a decreased number in response to higher BACs during the third or all three trimester-equivalents, but none of these alterations are observed when the exposure period does not include the third trimester-equivalent.

The Effects of Three Trimester-Equivalent Binge Alcohol Exposure on the Fetal Hippocampal Pyramidal Cells

Our study also demonstrates no alteration in the total number of hippocampal CA1 and CA2/3 field pyramidal cell numbers in response to all three trimester-equivalent binge alcohol exposure in fetal sheep. A careful comparison with the neuroanatomical studies conducted using the rat model reveals that these findings are not different from earlier reports. Miller (1995) demonstrated no reductions in CA1 pyramidal cell number in response to third trimester-equivalent alcohol (BAC, 222 mg/dl) and decreases at much higher does (BAC, 339 mg/dl). Similarly, Bonthius and West (1990) demonstrated decreased CA1 pyramidal cells in response to postnatal alcohol exposure at the highest dose (BAC, 361 mg/dl), while no such reduction was found in response to a BAC of 190 mg/dl. The recent study by Livy and coworkers also

demonstrated decreased number of CA1 and CA3 pyramidal cells in response to high doses of alcohol (BAC, 339 mg/dl), but these effects were again not observed when the exposure period did not include the third trimester-equivalent (Livy et al., 2003). These findings are further supported by another study where first two trimester-equivalent alcohol exposure did not result in any difference between the alcohol and the control groups (Maier and West, 2001b). Taken together, all three trimester-equivalent exposure to moderate doses of alcohol does not alter the number of pyramidal cells in the developing hippocampus, whereas exceptionally high BACs may selectively reduce CA1 pyramidal cell number. Interestingly, we recently reported that the estimated number of cerebellar Purkinje cells were reduced in response to all three trimester-equivalent alcohol exposure (BAC, 189 mg/dl) in the sheep model (Ramadoss et al., 2007b), a finding taken together with this report demonstrates the highly selective actions of alcohol on the fetal brain.

CONCLUSIONS

We conclude that all three trimester-equivalent binge alcohol exposure *in utero* results in a selective increase in the density of the granule cells of the fetal dentate gyrus, without any change to its overall number. We also demonstrate that a moderate dose of alcohol administered throughout gestation alters neither CA2/3 nor CA1 pyramidal cell numbers in the fetal hippocampus. However, rodent FASD studies clearly show that exceptionally high BACs may reduce the numbers of all cell types within the hippocampal formation. Therefore, our study suggests that impairments associated with the hippocampal function and behavioral modifications related to learning and memory in children prenatally exposed to alcohol may result from decreased number of neurons in the hippocampal formation only if they were exposed to very high BACs.

6. ALCOHOL INDUCED ACIDOSIS AND GLUTAMINE

INTRODUCTION

The teratogenic effects of ethanol, collectively referred to as Fetal Alcohol Spectrum Disorder (FASD), include physical, mental, behavioral and/or learning deficits of which Fetal Alcohol Syndrome (FAS), a severe form of FASD, is the most well known consequence (Riley and McGee, 2005; Sokol et al., 2003). Although considerable efforts have been made to educate women to not drink during pregnancy, the incidence of prenatal ethanol exposure has not declined (Caetano et al., 2006; CDC, 2004; NIAAA, 2000; Stratton et al., 1996). Currently, there are no known effective treatments, though some amelioration has been reported in a few individuals (Institute of Medicine, 1996).

A cardinal feature of FAS is growth deficiency (Sokol and Clarren, 1989). Prenatal ethanol exposure retards both growth and development of the fetus and progeny in rats (Lee and Leichter, 1983; Leichter and Lee, 1979). In children, prenatal ethanol exposure has been associated with growth retardation that can persist through 14 years of age (Day et al., 2002), suggesting that ethanol exposure during a sensitive or critical period of development has long-term implications for the offspring. The mechanisms by which *in utero* ethanol exposure induces fetal growth deficits are not well understood though fetal malnutrition is considered a major candidate. Ethanol could lead to fetal undernutrition by at least three possible ways: reduced maternal dietary intake (Schenker et al., 1990), impaired intestinal and/or placental transport of specific nutrients (Fisher et

al., 1981; Henderson et al., 1981; Lin, 1981; Polache et al., 1996), and altered maternal and/or fetal metabolism and compartmentalization of nutrients (Schenker et al., 1990). The consequences of any of these mechanisms by which the fetus is deprived of nutrients could lead to altered development and programming (Barker, 1994; Wu et al., 2004).

Normal fetal growth and development depend on a continuous supply of amino acids from the mother to the fetus. A number of amino acids have been demonstrated to be reduced in the maternal and the fetal compartments in response to gestational ethanol exposure in rodents. Acute ethanol exposure in the pregnant mouse results in a significant reduction in the plasma concentrations of threonine, serine, glutamine, glycine, alanine, and methionine (Padmanabhan et al., 2002). Chronic ethanol exposure during the first two trimester-equivalents of human brain growth (12) modeled in the rat has been shown to reduce maternal plasma proline concentration (Marquis et al., 1984). Utilizing a chronic paradigm during the first two trimester-equivalents of human brain growth, Karl and coworkers (Karl et al., 1995) found altered plasma glutamate in the fetal rat, but not in the mother. However, none of these studies have provided a coherent explanation for ethanol induced alterations in plasma concentrations of amino acids during pregnancy.

A series of studies from our laboratory have demonstrated that with each bout of maternal ethanol exposure, the mother and the fetus experience transient increases in arterial partial pressure of carbon dioxide (P_aCO_2), resulting in a reduction in arterial pH (pH_a) (Cudd et al., 2001b; Ramadoss et al., 2006b; Ramadoss et al., 2007b; West et al.,

2001). Acute pH changes are known to alter glutamine/glutamate metabolism and transport across cell membranes (Curthoys and Watford, 1995; Nissim, 1999). Glutamine is a major nutrient required by the fetus for growth (Kwon et al., 2003), and a reduction in its maternal plasma concentration might be detrimental to the normal growth and development of the fetus. Moreover, a disturbance in glutamine homeostasis could also lead to alterations in the levels of several other amino acids like arginine and citrulline whose biosyntheses depend on glutamine. Therefore, we hypothesized that ethanol mediated acidosis is a candidate mechanism governing alterations in maternal amino acid homeostasis, and that by creating the same magnitude and pattern of acidemia, independent of ethanol exposure, in pregnant ewes (see "Critique of the methods" below for the rationale for choosing this animal model) as that produced by ethanol would result in similar alterations in the circulating concentrations of glutamine and the amino acids that are glutamine-dependent. The experimental paradigm was designed to study the response to an acute dose of ethanol, following four weekly three day binge exposures (a drinking pattern common in women who use ethanol during pregnancy) (Caetano et al., 2006; Cudd et al., 2001a; Ebrahim et al., 1999; Gladstone et al., 1996; Maier and West, 2001), lasting the third trimester-equivalent of human brain development in sheep, a period of high velocity brain growth when it is known that the brain is sensitive to ethanol mediated damage (Cudd, 2005; Dobbing and Sands, 1979).

METHODS

Subjects

The experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University. Suffolk ewes, aged 2-6 years of age, were mated and pregnancies of known date of conception were confirmed as previously described (Ramadoss et al., 2006a). In brief, time dated pregnancies were achieved by controlling the estrous cycle through the use of progesterone impregnated vaginal implants (EAZI-BREED™, CIDR®, Pharmacia & Upjohn Ltd., Auckland New Zealand). Implants were removed 11 days after placement at which time prostaglandin $F_{2\alpha}$ (20 mg; LUTALYSE®, Pharmacia & Upjohn, Kalamazoo MI) was intramuscularly administered. The following day, ewes were placed with a ram fitted with a marking harness for a period of 24 hours. Marked ewes were assessed ultrasonographically on gestational days (GD) 25, 60 and 90 to confirm pregnancy. Ewes were maintained in shaded outdoor pens with herdmates from before mating until GD 100. On GD 100, the ewes were relocated to an environmentally regulated facility (22ºC and a 12:12 light/dark cycle) where they remained for the duration of the experiments. Animals in all treatment groups were fed 2 kg/day of a "complete" ration (Sheep and Goat Pellet, Producers Cooperative, Bryan, TX). All animals consumed all of the feed offered.

Experiment Protocol

Animals were divided into three experimental groups: an ethanol group, an acidemic group, and a pair-fed saline control (SC) group. A week before the start of the

experiment, on GD 102, the ewes underwent surgery to chronically implant femoral arterial and venous catheters (0.050" inner diameter, 0.090" outer diameter polyvinyl chloride). Details of the surgery protocol have been described earlier (Cudd et al., 2001b). The experiments were conducted on three consecutive days beginning on GD 109 followed by 4 days without treatment with the weekly pattern being repeated until GD 132. In all treatment groups, the infusion solutions were delivered intravenously over an hour by peristaltic pump (Masterflex, model 7014-20, Cole parmer, Niles IL) through a 0.2 µm bacteriostatic filter. Pumps were calibrated before infusion. Ewes in the ethanol treatment group received ethanol at a dosage of 1.75 g/kg body weight, as a 40% w/v solution diluted in 0.9% saline. Subjects in the pair-fed saline control and the acidemic groups received saline (0.9% saline) at a rate and volume equivalent to that of the ethanol group on a per kg basis.

On the day of an experiment, ewes were placed in a modified metabolism cart so that the animal's head was inside a plexiglass chamber (Figure A-16). A vinyl diaphragm attached to the open side of the chamber was drawn around the animal's neck to isolate the atmosphere in the chamber from ambient air. In the acidemic group, subjects were exposed to increased inspired fractional concentrations of carbon dioxide for 6 hours, to create a similar magnitude and pattern of reduction in the arterial pH compared to that produced by ethanol in previous studies (Cudd et al., 2001b) (see "Critique of the methods" for the rationale for using respiratory manipulation of arterial pH). The rate at which CO_2 was introduced into the chamber in the acidemic group was determined by monitoring maternal arterial pH; the CO_2 inflow rate was adjusted so that

maternal arterial pH in the acidemic and ethanol groups were matched over the duration of the 6 hour experimental period on all 12 experimental days. The percentage of oxygen and carbon dioxide in the chamber was measured using a gas monitor (oxygen, model S-3A; carbon-dioxide, model CD-3A, Applied Technologies, Pittsburgh, PA). Normoxemic conditions were maintained throughout the experiment in the acidemic group. Subjects in the ethanol and the saline groups had their heads inside the plexiglass chamber, but the chamber bottom was removed to allow breathing of room air.

Blood (1 ml) was drawn from the femoral artery catheter at 0, 0.5, 1, 1.5, 2, 3, 4, 5, and 6 hours for blood gas analysis on all experiment days. Samples were collected in heparinized 3 ml syringes, capped and immediately analyzed using a blood gas analyzer (ABL 5; Radiometer, Westlake, OH). Blood ethanol concentration (BEC) was measured at 0 and 1 hour. A 20 μl aliquot of blood was collected into microcapillary tubes and transferred into vials that contained 0.6 N perchloric acid and 4 mM n-propyl ethanol (internal standard) in distilled water. The vials were tightly capped with a septum sealed lid and were stored at room temperature until analysis by headspace gas chromatography (Varian Associates, model 3900, Palo Alto, CA) at least 24 hr after collection. The basic gas chromatographic parameters were similar to those reported by Penton (Penton, 1985), with the exception of the column (DB-wax, Megabore, J&W Scientific Folsum, CA) and the carrier gas (helium) used (West et al., 2001).

Assessment of plasma amino acid, lactate, urea and glucose concentrations in response to the acute challenge following chronic exposure was performed on GD 132, the last day of the study, by withdrawing blood samples (1 mL) at the beginning and at

the end of the infusion (1 hour), the time at which peak BEC is achieved (Cudd et al., 2001b). Samples were immediately centrifuged for 10 minutes at 3000 x g and 4°C. Plasma was acidified with 1 mL of 1.5 mol/L $HClO_4$ and then neutralized with 0.5 mL of 2 mol/L K_2CO_3. The supernatant was used for amino acid analysis by HPLC, as previously described (Wu et al., 1997). Plasma levels of glucose, lactate, and urea were analyzed by spectrophotometric enzymic methods (glucose, using hexokinase and glucose 6P dehydrogenase; urea, using urease; lactate, using lactate dehydrogenase) as previously described (Fu et al., 2005; Wu et al., 1995).

Statistical Analyses

The percentage change in the levels of the nutrients/metabolites at one hour (the end of ethanol/saline infusion) compared with that at 0 hour (baseline), were analyzed using a one-way ANOVA with treatment group as the sole dependent factor. Post-hoc tests were conducted using Fishers protected LSD test. The α level was established *a priori* at $p < 0.05$ for all analyses; p values between 0.05 and 0.10 were considered trends.

RESULTS

The saline control, ethanol, and acidemia groups consisted of 6, 5 and 6 subjects respectively and the data from all subjects were used for analysis of blood gases, plasma amino acids.

Maternal Arterial Blood Gases and Lactate Levels

Arterial pH at the 1st hour after the beginning of the treatment was reduced in the ethanol and the acidemic groups, compared to the pair-fed saline control group and these decreases persisted for 5 hours after the end of infusion (Figure A-17). The magnitude of pH reduction in the acidemic group was nearly identical to that in the ethanol group at all time points. This nearly identical decrease in the maternal arterial pH in the acidemic group was created by increasing the inspired partial pressure of carbon dioxide, independent of ethanol. Maternal P_aCO_2 similarly peaked at the 1st hour in the ethanol and the acidemic groups compared to the pair-fed saline control group, while measurements at 0th hour were not different among groups (baseline P_aCO_2, SC, 34 $\pm$ 0 mm Hg; ethanol, 33 $\pm$ 0 mm Hg; acidemia, 34 $\pm$ 1 mm Hg). P_aCO_2 values at the 1st hour were 35 $\pm$ 0 mm Hg, 39 $\pm$ 0 mm Hg, and 52 $\pm$ 1 mm Hg in the saline control, ethanol, and acidemia groups respectively. Lactate levels were elevated at the 1st hour in the ethanol group (~ 100%), but not in the acidemic or the pair-fed saline control group (Figure A-18), explaining why a greater P_aCO_2 elevation was required to produce a similar magnitude of acidemia as that in the ethanol group. Consequently, ethanol mediated acidemia had both a respiratory and a metabolic component associated with it,

while the acidemia group was not subjected to metabolic acidosis. The maternal arterial partial pressure of oxygen (P_aO_2) at the 0^{th} hour was 97 $\pm$ 1 mm Hg, 90 $\pm$ 1 mm Hg, and 87 $\pm$ 2 mm Hg in the saline control, ethanol, and acidemia groups respectively. P_aO_2 decreased significantly in the ethanol group to 83 $\pm$ 1 mm Hg at the 1^{st} hour, but no such reduction was found in the saline control or the acidemic groups.

Plasma Glucose and Urea

No differences were detected in the maternal plasma glucose level at the beginning of the last day of treatment (SC, 45 $\pm$ 3 mg/dl; ethanol, 46 $\pm$ 5 mg/dl; acidemia, 53 $\pm$ 4 mg/dl) or at the end of the last acute challenge among treatment groups (Figure A-18). Plasma urea concentrations were not different when comparing among groups.

Maternal Plasma Concentrations of Amino Acids

Maternal plasma concentrations of most amino acids at the beginning of the last day of the experiment were not different among groups (Table B-1). However, some differences were noted: glutamine and glutamate were significantly reduced in the ethanol group; arginine, asparagine, and serine were significantly elevated in the acidemic group, while branched chain amino acids (BCAAs) were significantly higher in the ethanol as well as the acidemic groups, compared with that in the pair-fed saline control group.

A significant reduction in the maternal plasma concentrations of amino acids in response to an acute exposure following the chronic exposure was observed in the ethanol and the acidemic groups compared with those in the pair-fed saline control group (Figure A-19). The reductions in the levels of arginine (~ 42%), citrulline (~ 25%), asparagine (~ 44%), serine (~ 30), threonine (~ 44%), tryptophan (~ 24%), methionine (~ 46%), leucine (~ 31%), histidine (~ 14%), tyrosine (~ 24%), valine (~ 53%), and isoleucine (~ 35%) were significant at one hour in the ethanol group. The same specific amino acids were reduced significantly in the acidemic group. Glutamine was significantly reduced (~ 40%) in the ethanol group, whereas it trended lower (~ 25%) in the acidemic group. The only amino acid that exhibited a significant increase in response to ethanol was glutamate (~ 58%). In the acidemia group, glutamate was elevated (~ 30%) but the response was not statistically significant. Post-hoc analysis did not identify differences between the ethanol and acidemia groups for any of these amino acid concentrations. In contrast, glycine, taurine, alanine, ornithine, and phenylalanine were not different among groups. The pair-fed saline control group exhibited no alterations in any of the amino acids at the 1st hour, compared with baseline values.

DISCUSSION

Critique of the Methods

Rationale for the pattern and timing of exposure

We used a chronic weekly-weekend binge ethanol exposure paradigm, a common pattern of drinking in women who abuse ethanol during pregnancy (Caetano et al., 2006; Cudd et al., 2001a; Ebrahim et al., 1999; Gladstone et al., 1996; Maier and West, 2001a). In humans, the velocity of fetal brain growth increases throughout the third trimester to peak at parturition (33). This period of third trimester high velocity brain growth is a time when it is known that there is a high sensitivity to ethanol mediated brain damage (Dobbing and Sands, 1979; West et al., 2001). In order to extrapolate this timing of exposure from sheep to humans, we differentiated the equation reported by Richardson and Herbert that predicts ovine fetal brain weight as a function of gestational age, with respect to time, and the first order velocity curve was plotted similar to that of Dobbing and Sands (Dobbing and Sands, 1979; Richardson and Hebert, 1978) (Figure A-20). In the sheep, the velocity of brain growth declines significantly after GD 132. Therefore, the experiment was performed in pregnant sheep from GD 109 to GD 132 (term is 147 days) in order to best model the period of human third trimester high velocity brain growth.

Rationale for the use of the sheep model

The sheep was chosen because the third trimester equivalent of brain development in this species occurs *in utero*, as it does in the human and not postnatally

as occurs in rats. Using a postnatal model of third trimester-equivalent of human brain development like the rat or mouse would add the potential confounds of eliminating maternal, placental and fetal interactions or a role of parturition in mediating the damage. In addition, we have previously established that the third trimester equivalent in sheep is a period of increased vulnerability to ethanol as it is in humans and in other animal models (Livy et al., 2003; West et al., 2001). Further, the sheep was utilized because of the robustness of this species in experiments requiring chronic instrumentation. We chose not to instrument the fetuses because the studies lasted from GD 109 until GD 132, the end of the third trimester equivalent of human brain development. Instrumenting the fetuses before the beginning of the experiments (in order to allow recovery from surgery) and then extending the preparations until GD 132 would have involved unacceptable wastage with respect to addressing our primary question, the effects of chronic ethanol and pH changes on maternal amino acid concentrations.

Rationale for using a respiratory manipulation to create acidemia

We hypothesized that alcohol induced maternal acidosis would alter maternal glutamine homeostasis. Ethanol exposure results in mixed respiratory and metabolic acidosis in the mother and fetus (Cudd et al., 2001b; Gleason and Hotchkiss, 1992; Ramadoss et al., 2007d). We mimicked the pH pattern produced by ethanol by manipulating maternal P_aCO_2 rather than by using an exclusively metabolic or a mixed respiratory/metabolic approach for the following reasons: 1) altering CO_2 affects pH in

all fetal and maternal body fluid compartments and 2) precise manipulation of arterial pH could be maintained over 6 hours by respiratory means without the potential confounds of creating fluid volume or electrolyte changes that might occur in response to prolonged acid infusions. And finally, it has been demonstrated in other animal model systems that the renal glutamine uptake in respiratory and metabolic acidosis is comparable (Gougoux et al., 1982; Windus et al., 1984).

Ethanol Alters Maternal Plasma Amino Acid Levels

To our knowledge, this is the first study to identify dynamic changes in maternal plasma concentrations of amino acids in response to an acute challenge of ethanol following a chronic exposure throughout the third trimester-equivalent of human brain growth. The maternal plasma amino acid concentrations in this study in the pair-fed saline control group were similar to those reported previously in normal pregnant ewes on a similar day of gestation (Kwon et al., 2003). The concentrations of most amino acids at the beginning of the last day of experiment were not different among the treatment groups though glutamine and glutamate concentration were reduced in the ethanol group and BCAA concentrations were elevated in the ethanol and the acidemic groups. A final acute challenge of ethanol or acidemia following the chronic exposure resulted in a significant reduction of glutamine, arginine, citrulline, asparagine, serine, threonine, tryptophan, methionine, leucine, histidine, tyrosine, valine, and isoleucine, compared with that at baseline, while glutamate was elevated. Glucose, in contrast, was not altered in response to ethanol, a finding that is consistent with previous reports;

maternal ethanol consumption reduces fetal, but not maternal plasma glucose levels (Falconer, 1990; Marquis et al., 1984; Richardson et al., 1985). Utilizing a chronic, but not a binge ethanol exposure during the first two trimester-equivalents of human brain growth in rats, Karl and coworkers (Karl et al., 1995) found increased fetal glutamate levels, but glutamine was not altered in the mother or the fetus. Other rat studies involving a similar exposure paradigm have demonstrated a decrease in maternal plasma proline levels (Marquis et al., 1984), as well as reduced sodium dependent intestinal absorption of methionine (Polache et al., 1996), and taurine (Martin-Algarra et al., 1998). In contrast to these chronic exposure studies, an acute ethanol study in the pregnant mouse resulted in a significant reduction in the plasma concentrations of several amino acids, including threonine, serine, glutamine, glycine, alanine, and methionine (Padmanabhan et al., 2002). The potential adverse effects of these decreases in maternal amino acid levels would be further compounded by ethanol mediated reductions in transplacental amino acid transport (Fisher et al., 1981; Henderson et al., 1981; Lin, 1981; Schenker et al., 1990), leading to further decreased availability of fetal nutrients.

Acidemia Is a Central Mechanism Governing Ethanol Mediated Alterations in Amino Acid Levels

Ethanol induced a mixed respiratory and metabolic acidosis in this study. This finding is consistent with previous reports from this laboratory where we have shown that with each bout of ethanol, the mother and the fetus are subjected to transient

hypercapnea and acidemia, irrespective of the period of gestation (Cudd et al., 2001b; Ramadoss et al., 2007b). The current study extends our previous work by demonstrating that an acute acidemic challenge (independent of ethanol), of a nearly identical magnitude to that brought about by ethanol, resulted in similar amino acid alterations as in the ethanol treatment group. The same specific amino acids were altered in both groups suggesting that acidemia is the central mechanism mediating these changes in amino acid concentrations. We propose the following model to explain the amino acid dynamics in ethanol induced acidemia.

The Ethanol-Glutamine Model

The role of the kidney

The maternal kidney is one of two organ systems that are largely responsible for alterations in maternal amino acid levels in response to acidosis (Figure A-21). In the present study, we observed a significant decrease in glutamine levels in response to an acute challenge following the chronic exposure, a finding that is consistent with previous reports where acidosis has been shown to decrease plasma glutamine levels (Heitmann and Bergman, 1980) due to increased renal glutamine uptake, by 365% in nonpregnant sheep (Heitmann and Bergman, 1980), and by even higher values in humans and rats (Welbourne, 1987; Welbourne et al., 1986). The kidneys extract and catabolise more than one third of plasma glutamine in a single pass through the organ (Taylor and Curthoys, 2004). Twenty percent of glutamine is known to be filtered and reabsorbed and, within 1 to 3 hours of acidosis, renal extraction of plasma glutamine exceeds the

percent filtered by glomeruli (Hughey et al., 1980; Taylor and Curthoys, 2004). Several factors contribute to this increased renal absorption of plasma glutamine (Brosnan, 1987). First, acidosis stimulates glutamine uptake by renal mitochondria and its metabolism via the phosphate-dependent glutaminase (PDG) pathway (Nissim, 1999). Second, acidosis elicits increased expression of the system N glutamine transporter SNAT3 (SLC38A3) in basolateral membrane of the late proximal tubule S3 segment (Moret et al., 2007). This alteration in the renal glutamine transporter is glucocorticoid dependent (Karinch et al., 2007). Previous studies conducted in our laboratory have demonstrated that ethanol increases fetal as well as maternal cortisol levels (Cudd et al., 2001a). Therefore, these increases in cortisol would be expected to increase the glutamine uptake by the kidneys (Karinch et al., 2007), further contributing to the observed decreases in plasma glutamine levels in acidotic ewes. Third, formation of glutamine from glutamate and ammonia is negligible in the kidneys, especially in acidosis (Nissim, 1999). Taken together, these three processes explain the decreased plasma glutamine levels. Even though the gut and liver of the sheep also play a role in utilizing plasma glutamine, the intestinal extraction does not change in response to acidosis and the hepatic uptake of glutamine decreases in chronic acidosis (Heitmann and Bergman, 1980). It is worthy of note that in humans, the kidney is the only organ that is involved in plasma glutamine extraction during acidosis (Welbourne, 1987).

A reduction in plasma glutamine concentration will directly affect the levels of several other amino acids that are synthesized from glutamine (Wu and Morris, 1998); these include ornithine, citrulline (an intermediate in arginine synthesis from glutamine and proline), and arginine (Wu and Morris, 1998). In addition, elevated levels of lactate can reduce intestinal synthesis of citrulline and arginine from proline by inhibiting proline oxidase activity (Dillon et al., 1999). The glutamate formed from glutamine in the kidney is removed by glutamate dehydrogenase, by transamination reactions, and by transport from the intracellular (ICF) to the extracellular (ECF) compartment (Nissim, 1999), explaining the observed increases in plasma glutamate level in this study. Finally, an acidemia mediated increase in expression of renal glutamine SNAT3 transporter would result in increased renal uptake of histidine and asparagine, probably accounting for their decreased availability in maternal plasma (Chaudhry et al., 2001; Karinch et al., 2002).

The role of the skeletal muscle

The major source of plasma glutamine in sheep and humans is skeletal muscle (Welbourne, 1987). Some evidence suggests that glutamine release from muscle is not altered in response to acidosis in sheep (Heitmann and Bergman, 1980), a finding that would explain the observed decreases in plasma glutamine concentration. In the presence of ethanol mediated increases in glucocorticoids (Cudd et al., 2001a), acidosis would up-regulate the transamination of BCAAs with α-ketoglutarate to form glutamate and branched chain α-ketoacids (BCKAs) and would also directly stimulate the oxidative catabolism of BCKAs through the activation of BCKA dehydrogensase, ultimately leading to decreased plasma levels of BCAAs (May et al., 1987). Interestingly, plasma concentrations of BCAAs at the beginning of the last ethanol or acidemia treatment were elevated (Table B-1), suggesting a decrease in their intramuscular catabolism or an increase in net proteolysis.

CONCLUSION AND PERSPECTIVES

Results of this study support the conclusion that chronic binge ethanol induced acidosis alters amino acid homeostasis in pregnant ewes. These novel findings may help explain intra-uterine growth retardation and structural damage to the nervous system observed in FASD. Women who drink during pregnancy may exhibit decreases in plasma amino acid concentrations and thus availability of glutamine and related amino acids with each bout of ethanol consumption. Repeated perturbations in the fetal availability of specific nutrients due to altered concentrations in maternal plasma could result in impaired development and altered programming with life-long consequences for the offspring. Therefore, we conclude that maternal acidosis and glutamine dependent pathways should be taken into consideration in the development of effective therapeutic measures for FASD.

7. EFFECTS OF ACIDOSIS ON THE CEREBELLUM

INTRODUCTION

Ethanol abuse during pregnancy can result in a variety of deleterious effects that include physical, mental, behavioral, and/or learning deficits, collectively referred to as Fetal Alcohol Spectrum Disorders (FASD) (Riley and McGee, 2005; Sokol et al., 2003). Heavy maternal drinking is the leading cause of birth defects in the western world, accounting for 1 case of FASD per 100 live births (Sokol et al., 2003; Stratton et al., 1996). Broad public health measures to prevent maternal ethanol consumption have met with limited success (Cudd, 2005; Stratton et al., 1996); the incidence of FASD has not decreased (Caetano et al., 2006; CDC, 2004; NIAAA, 2000; Stratton et al., 1996). Therefore, in addition to education, ways to prevent or mitigate the effects of prenatal ethanol exposure must be explored (Cudd, 2005).

Fetal cerebral hypoxia is one of the most cited mechanisms for the teratogenic effects of ethanol. However, the experimental evidence supporting ethanol-induced cerebral hypoxia as a mechanism of FASD is largely based on indirect observations. For example, both hypoxia and ethanol exposure during development induce similar craniofacial and neuronal deficits (Aitken and Schiff, 1986; Bronsky et al., 1986). More recent studies have demonstrated that ethanol exposure (1-3 g/kg) in sheep during the third trimester-equivalent does not result in fetal hypoxemia, despite a mild transient reduction in the maternal arterial partial pressure of oxygen (P_aO_2) to around 83 mm Hg (Cudd et al., 2001b; Falconer, 1990; Patrick et al., 1985; Richardson et al., 1985; Smith

et al., 1989a). In fact, chronic ethanol (1.75 g/kg) results in decreases in the fetal total peripheral vascular resistance, leading to increased cerebral blood flow and oxygen delivery (Parnell et al., 2007), a set of findings that do not support the cerebral hypoxia theory.

Though ethanol does not lead to fetal hypoxemia or hypoxia, it has been demonstrated to result in maternal and fetal hypercapnea and acidemia (Cudd et al., 2001b; Parnell et al., 2007; Ramadoss et al., 2007a; Ramadoss et al., 2007d), and interestingly fetal acidosis has been suggested to be a potential mechanism underlying the teratogenic effects of ethanol (Horiguchi et al., 1971) even before Fetal Alcohol Syndrome (FAS), was described by Jones and Colleagues (Jones et al., 1973). Severe brain acidosis has been documented to cause neuronal and glial death (Goldman et al., 1989; Kraig et al., 1987; Staub et al., 1990), and some candidate mechanisms include exacerbation of free radical mediated injury (Ying et al., 1999),), alteration of gene expression and protein synthesis by perturbing intracellular signal transduction pathways (Siesjo et al., 1996), decrease of serum IGF-1 concentrations (Brungger et al., 1997; Challa et al., 1993; Wiederkehr and Krapf, 2001), and increase of glucocorticoid levels (Perez et al., 1979; Wiederkehr and Krapf, 2001; Wood and Chen, 1989). We also recently reported that ethanol-induced acidemia decreases the concentrations of glutamine and glutamine-related amino acids, resulting in decreased bioavailability of nutrients to the fetus (Ramadoss et al., 2007d), while glutamate is elevated. Therefore, we speculated that chronic binge alcohol exposure mediated acidemia may be deleterious to fetal brain development.

Developmental third trimester-equivalent ethanol exposure is documented to result in loss of certain neuronal cell types in specific brain regions, especially the Purkinje and granule cells of the cerebellum (Goodlett and Eilers, 1997; Hamre and West, 1993; Ramadoss et al., 2007a; West et al., 2001). Therefore, we hypothesized that repeated ethanol exposures leading to acidemia, 3 days in succession per week (a common binge drinking paradigm) (Caetano et al., 2006; Ebrahim et al., 1999; Gladstone et al., 1996) throughout the third trimester would lead to fetal cerebellar Purkinje cell loss. The corollary hypotheses are that the ethanol-mediated transient reduction in maternal P_aO_2 and acidemia play a role or that blood gases do not play a role. The measurement of neuronal loss was conducted using a stereological cell counting technique.

A second goal in this study was to examine the cerebellum upon inhibition of ethanol-induced fetal acidemia by selective blockade of the novel TWIK acid sensitive potassium channels (TASK) expressed in the peripheral and central chemoreceptors (Duprat et al., 1997). TASK channels express a standing outwardly rectifying potassium current. They depolarize upon inhibition and thus increase firing rate to the respiratory control centers, and thus increase the tidal volume and the respiratory rate. (Han et al., 2002; Millar et al., 2000; Duprat et al., 1997). These experiments were performed in the sheep fetus during a period of high velocity brain growth like that during the third trimester in humans (Cudd, 2005), a period when it is known that the brain is sensitive to ethanol mediated damage (Maier et al., 1999).

METHODS

Subjects

The experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University. Suffolk ewes, aged 2-6 years of age, were mated and pregnancies of known date of conception were confirmed as previously described (Ramadoss et al., 2006a). The day of mating (the day that ewes were marked by the ram) was designated as gestational day (GD) 0. Ewes were maintained in shaded outdoor pens with herdmates from before mating until GD 90. On GD 90, the ewes were relocated to an environmentally regulated facility (22ºC and a 12:12 light/dark cycle) where they remained for the duration of the experiments. Animals in all treatment groups were fed 2 kg/day of a "complete" ration (Sheep and Goat Pellet, Producers Cooperative, Bryan, TX). All animals consumed all of the feed offered.

Treatment Groups

Nine treatment groups were used in this study (Table B-1): (1) an untreated normal control group, (2) a pair-fed saline control (received 0.9% saline at a rate and volume equivalent to that of the ethanol group on a per kg basis) that served as a control for nutrition, instrumentation and the volume of infusion delivered, (3) an ethanol group that received ethanol at a dosage of 1.75 g/kg body weight, as a 40% w/v solution diluted in 0.9% saline, (4) an ethanol-normoxemic (ethanol-normox) group, where a transient decrease in the maternal arterial partial pressure of oxygen (P_aO_2) produced at

the end of each ethanol infusion was abolished by providing increased inspired fractional concentration of oxygen, (5) an acidemic-normoxemic (acidemic-normox) group, where the arterial pH (pH_a) pattern produced by ethanol was mimicked for the whole of the third trimester-equivalent independent of ethanol (received saline), (6) an acidemic-hypoxemic (acidemic-hypox) group, where the maternal fall in pH_a and the mild decrease in maternal P_aO_2 was mimicked, (7) an ethanol-TASK inhibitor (ethanol-TI) group, where the ethanol-induced acidemia was prevented by modulating pathways that control respiration, (8) a saline-TASK inhibitor (saline-TI) group that served as a control for the ethanol-TI group, (9) and a fetal instrumented group, in order to measure fetal blood gases. The fetuses were not instrumented in the first six treatment groups because the studies lasted from GD 109 until GD 132, the end of the third trimester equivalent of human brain development. Instrumenting the fetuses before the beginning of the experiments (in order to allow recovery from surgery) and then extending the preparations until GD 132 would have involved unacceptable wastage with respect to addressing our question, the effect of alcohol-induced alterations in maternal blood gases on fetal brain. The mothers in the fetal instrumented group were randomized so that subjects received each of the four treatments (saline, alcohol, acidemia-normox, or acidemia-hypox) on GD 118, GD 123, GD 125, and GD 132.

Maternal and Fetal Surgery

In all groups, except the normal control and fetal instrumented groups, a week before the start of the experiment, on GD 102, the ewes underwent surgery to

chronically implant femoral arterial and venous catheters (0.050" inner diameter, 0.090" outer diameter polyvinyl chloride). Details of the surgery protocol have been described earlier (Cudd et al., 2001b).

In the fetal instrumented group, surgery was performed on GD 113 to implant chronic indwelling catheters as previously described (Cudd et al., 2001b). In brief, a ventral midline laparotomy was performed and the uterus and fetal membranes were incised. A catheter (0.030" inner diameter, 0.050" outer diameter polyvinyl chloride) was passed from the cranial tibial artery into the abdominal aorta. Catheters were passed through the flank of the ewe and were stored in a pouch attached to the skin.

Experiment Protocol

The experiments were conducted on three consecutive days beginning on GD 109 followed by 4 days without treatment with the weekly pattern being repeated until GD 132. In all treatment groups, the infusion solutions were delivered intravenously over an hour by peristaltic pump (Masterflex, model 7014-20, Cole parmer, Niles IL) through a 0.2 μm bacteriostatic filter. Pumps were calibrated before infusion.

On the day of an experiment, ewes were placed in a modified metabolism cart so that the animal's head was inside a plexiglass chamber (Ramadoss et al., 2007d). A vinyl diaphragm attached to the open side of the chamber was drawn around the animal's neck to isolate the atmosphere in the chamber from ambient air (Table B-1). In the acidemic (acidemic-normox and acidemic-hypox) groups, subjects were exposed to increased inspired fractional concentrations of carbon dioxide for 6 hours, to create a

matching magnitude and pattern of reduction in the arterial pH (pH_a) compared to that produced by ethanol in previous studies (Cudd et al., 2001b) and in he present study. The rate at which CO_2 was introduced into the chamber in the acidemic groups was determined by monitoring maternal arterial pH_a (ABL 5, Radiometer, Cleveland, OH); the CO_2 inflow rate was adjusted so that maternal pH_a in the acidemic and ethanol groups were matched over the duration of the 6 hour experimental period on all 12 experimental days. The percentage of oxygen and carbon dioxide in the chamber was measured using a gas monitor (oxygen, model S-3A; carbon-dioxide, model CD-3A, Applied Technologies, Pittsburgh, PA). Normoxemic conditions were maintained throughout the experiment in the acidemic-normox group. In the acidemic-hypox group, in addition to replicating the acidemia, the mild transient reduction in the maternal P_aO_2 observed at the 1st hour in response to ethanol (the end of ethanol infusion) (Cudd et al., 2001b) was mimicked by increasing the inspired fractional concentration of nitrogen. In the ethanol-normox group, the transient reduction in maternal P_aO_2 was abolished by increased inspired fractional concentration of oxygen. Subjects in the ethanol and the saline control groups had their heads inside the plexiglass chamber, but the chamber bottom was removed to allow breathing of room air. In the ethanol-TI group, a selective TASK channel blocker (Doxapram hydrochloride, Dopram-V, 20 mg/mL, Fort Dodge, Iowa) was administered to prevent the ethanol-induced decreases in pH. The rate at which TI was infused was empirically adjusted by withdrawing maternal blood samples every 15 minutes for 6 hours on every day of the experiment.

Manipulation of Maternal Acid-Base Status

Blood (1 ml) was drawn from the femoral artery catheter at 0, 0.5, 1, 1.5, 2, 3, 4, 5, and 6 hours for blood gas analysis on all experiment days. Samples were collected in heparinized 3 ml syringes, capped and immediately analyzed using a blood gas analyzer (ABL 5; Radiometer, Westlake, OH). Arterial pH (pH_a) at the 1st hour after the beginning of the treatment was reduced in the ethanol, ethanol-normox, acidemic-normox, and the acidemic-hypox groups, compared to the pair-fed saline control, ethanol-TI, and saline-TI groups and these decreases persisted for 5 hours after the end of infusion (Figure A-22). The fall in pH_a induced by ethanol was inhibited administering TI in the ethanol-TI group. Further, the magnitude of pH_a reduction in both the acidemic groups was nearly identical to that in the two ethanol groups at all time points. This nearly identical decrease in the maternal arterial pH in the acidemic group was created by increasing the inspired partial pressure of carbon dioxide, independent of ethanol. Maternal P_aCO_2 similarly peaked at the 1st hour in the ethanol and the acidemic groups compared to the pair-fed saline control group, while measurements at 0th hour were not different among groups (baseline P_aCO_2, saline control, 34 $\pm$ 0 mm Hg; ethanol, 32 $\pm$ 1 mm Hg; ethanol-normox, 34 $\pm$ 0 mm Hg; acidemic-normox, 34 $\pm$ 1 mm Hg; acidemic-hypox, 34 $\pm$ 0 mm Hg; ethanol-TI, 34 $\pm$ 1 mm Hg; saline-TI, 34 $\pm$ 1 mm Hg). P_aCO_2 values at the 1st hour were 35 $\pm$ 0 mm Hg, 40 $\pm$ 1 mm Hg, 40 $\pm$ 1 mm Hg, 45 $\pm$ 1 mm Hg, 52 $\pm$ 1 mm Hg, 34 $\pm$ 1 mm Hg, and 32 $\pm$ 1 mm Hg in the saline control, ethanol, ethanol-normox, acidemic-normox, acidemic-hypox, ethanol-TI, and saline-TI groups respectively. Maternal lactate levels were

increased (by ~ 100%) only in the ethanol groups, and not in response to acidemia produced by hypercapnea, explaining why a greater P_aCO_2 elevation was required to produce a similar magnitude of acidemia as that in the ethanol group. Consequently, ethanol mediated acidemia had both a respiratory and a metabolic component associated with it, while the acidemia groups were not subjected to metabolic acidosis. The maternal arterial partial pressure of oxygen (P_aO_2) at the 0^{th} hour was 96 $\pm$ 1 mm Hg, 90 $\pm$ 1 mm Hg, 97 $\pm$ 1 mm Hg, 91 $\pm$ 2 mm Hg, 91 $\pm$ 1 mm Hg, 99 $\pm$ 2 mm Hg, and 96 $\pm$ 3 mm Hg in the saline control, ethanol, ethanol-normox, acidemic-normox, and acidemic-hypox, ethanol-TI, and saline-TI groups respectively. P_aO_2 decreased in the ethanol group to around 85 mm Hg at the 1^{st} hour in ethanol, and acidemic-hypox groups, but no such reduction was found in the other treatment groups. Further, no biologically significant alterations were seen in P_aO_2 in any of the groups at any other time point.

Blood ethanol concentration (BEC) was measured at 0 and 1 hour. A 20 µl aliquot of blood was collected into microcapillary tubes and transferred into vials that contained 0.6 N perchloric acid and 4 mM n-propyl ethanol (internal standard) in distilled water. The vials were tightly capped with a septum sealed lid and were stored at room temperature until analysis by headspace gas chromatography (Varian Associates, model 3900, Palo Alto, CA) at least 24 hr after collection. The basic gas chromatographic parameters were similar to those reported by Penton, with the exception of the column (DB-wax, Megabore, J&W Scientific Folsum, CA) and the carrier gas (helium) used (Penton, 1985).

Fetal Cerebellar Tissue Processing

On GD 133, the ewes were euthanized using sodium pentobarbital (75 mg/kg, intravenously), and the fetuses were removed from the uterus and perfused with saline followed by cold fixative solution containing 1.25% paraformaldehyde and 3% glutaraldehyde in phosphate buffer (pH, 7.4). The brains were removed and stored in additional fixative until processed for stereological cell counting.

The cerebellum was dissected, embedded in 4% agar, and cut sagittally into five slabs. These slabs were dehydrated through increasing concentrations of ethanol (70, 95, 100%) and then infiltrated with increasing concentrations of infiltration solution (25, 50, 75, 100% methyl methacrylate; Technovit 7100^{TM} Embedding kit, Leica, Wetzlar, Germany). The tissue in each slab was embedded in a solution containing 1 ml dimethyl sulfoxide (hardener) per 15 ml of 100% infiltration solution and allowed to harden. After hardening, the tissue was sectioned into 30 μm sagittal sections by using a microtome (model RM2255, Leica, Nussloch, Germany). Every twentieth section was saved, mounted on a glass slide, stained with cresyl violet, and coverslipped.

Stereological Cell Counting

The total number of fetal cerebellar Purkinje cells was estimated using unbiased stereological cell counting techniques as described previously (Ramadoss et al.,

2007b). In brief, the Nikon (Garden City, NY) Optiphot microscope used in this study had a 40X objective lens with a 1.4 numerical aperture condenser. The microscope had a motor-driven stage to move within the x and y axes and an attached microcator to measure the z axis. The image was transferred to a personal computer (Millenium, Micron, Boise, ID) via a color video camera (model 2040, Jai, Copenhagen, Denmark). The reference volume was estimated using the Cavalieri's Principle and was calculated by the equation $V_{ref} = \Sigma p_i \text{ X } A(p_i) \text{ X } t$ where Σp_i is the total number of points (p_i) counted, $A(p_i)$ is the known area associated with each point, and t is the known distance between two serial sections counted. The GRID® software provided templates of points in various arrays that were used in point counting for reference volume estimation. The Purkinje cell density was determined by following the optical disector method, which was calculated using the formula $N_v = \Sigma Q / (\Sigma \text{disector X } A(fr) \text{ X } h)$ where ΣQ is the sum of the Purkinje cells counted from each disector frame, Σdisector is the sum of the

number of disector frames counted, A(fr) is the known area associated with each disector frame, and h is the known distance between two disector planes. The placement of the disector frames was determined by the GRID® software in a random manner. The estimated total number of Purkinje cells in the cerebellum was then calculated by multiplying the reference volume of the cerebellum and the numerical density of cells within this reference volume as described before (West et al., 2001).

Data Analysis

The stereology data were analyzed using a one-way ANOVA with "treatment" as the sole independent variable followed by protected Fishers LSD tests. Analyses of fetal arterial P_aO_2 and pH were performed using a mixed ANOVA with "treatment" as a between factor and "time" as a within factor. Statistical significance was established *a priori* at $p < 0.05$.

RESULTS

Ethanol and Fetal Acid-Base Status

The fetal arterial pH_a was significantly lower in the ethanol, acidemic-normox, and acidemic-hypox groups compared to the pair-fed saline control group (Figure A-23). Further, the pH_a at 1st hour was significantly lower than at all other time points. In contrast to these alterations in fetal pH_a, no such alteration in fetal arterial partial pressure of oxygen (P_aO_2) was found among the treatment groups or among time points.

Stereology Data

The estimated total number of fetal cerebellar Purkinje cells was significantly lower in the ethanol, and the ethanol-normox groups compared with that in the pair-fed saline control, and the normal control groups (Figure A-24). The mild transient decrease in the maternal P_aO_2 at the end of ethanol infusion was abolished in the ethanol-normox group, by increased inspired fractional concentration of oxygen; albeit this did not alter the number of Purkinje cells lost in response to ethanol. The acidemic-normox, and acidemic hypox groups also exhibited significantly lower number of cerebellar Purkinje cells compared to the two control groups. However, despite mimicking the fetal pH_a for 6 hours during the whole month third trimester-equivalent of chronic infusion, acidemia could not account for all of the cell loss; the number of cerebellar Purkinje cells in the acidemia groups was significantly different from the ethanol and ethanol-normox

groups. In the acidemic-hypox group, in addition to pH_a, the transient decrease in the maternal P_aO_2 at the 1st hour was mimicked. However, this did not result in any difference compared to when the pH_a alone was mimicked. This is understandable as the decrease in the maternal P_aO_2 at the 1st hour did not result in a similar decrease in fetal P_aO_2 in the ethanol group or the acidemic-hypox group. Most importantly, when ethanol was supplemented with TI, the ethanol-induced loss in cerebellar Purkinje cells was no more observed. Ethanol-TI group was not different from the normal and pair-fed saline control group. However. the ethanol-TI group was also significantly different from the ethanol, ethanol-normox and the two acidemia groups. When TI was administered with saline, no further significant increases in the Purkinje cell number was observed. The saline-TI group was not different from the control groups. No differences were noted between the two control groups, between the two ethanol groups, between the two acidemia groups, or between the two TI groups. Though the total number of

fetal cerebellar Purkinje cells was reduced in response to ethanol-induced acidemia, an overall statistical difference among groups in the cerebellar volume was not detected (Figure A-25). The cell density in the ethanol and acidemia groups was significantly lower compared with that in the normal control group (Figure A-26). The ethanol groups also had a significantly lower density compared to the saline control group. However, the acidemia group was not different compared to the saline control as well as the ethanol groups. When the ethanol group subjects were supplemented with TI, the ethanol-induced decrease in cerebellar Purkinje cell density was no more observed. TASK channel inhibition completely inhibited the teratogenic effects of ethanol on the fetal cerebellar Purkinje cells. No differences in cell density were detected between the controls, between the ethanol groups, between the acidemic groups, or between the TI groups.

DISCUSSION

The Cerebellum Is Vulnerable to Prenatal Ethanol Exposure

The present study used a quantitative stereological cell counting technique to demonstrate that chronic binge ethanol consumption during the third trimester-equivalent of human brain development results in a significant reduction in cerebellar Purkinje cell number in an ovine model, where all three trimester-equivalents occur *in utero*. FASD is characterized by central nervous system damage, at a gross, as well as microscopic level (for review, see Chen et al., 2003), and the affected children may exhibit abnormal development of the cerebellum (Archibald et al., 2001; Sowell et al., 1996; Swayze et al., 1997). Clinically, one of the most common abnormalities found in human prenatal ethanol exposure pathology studies has been the dysgenesis of cerebellum (Clarren et al., 1978; Peiffer et al., 1979; Wisniewski et al., 1983). MRI studies have clearly demonstrated a significantly smaller anterior cerebellar vermis in children who have been exposed to ethanol prenatally (Sowell et al., 1996), and it has been suggested that the cerebellum is the most sensitive morphological indicator of prenatal ethanol exposure in children (Autti-Ramo et al., 2002). Complementing these human studies, quantitative stereology studies conducted utilizing the rat model, further support this effect of ethanol on the developing cerebellum; it has been demonstrated that the cerebellum is more vulnerable to developmental ethanol exposure than any other brain structure and that the cerebellar Purkinje cells are more susceptible than any other cell type (Bonthius and West, 1991; Maier et al., 1997; Maier et al., 1999). The findings from the present study are in agreement with reports on humans and rodent models, that

the cerebellar Purkinje cells are vulnerable to alcohol exposure during the third trimester-equivalent of human brain development.

Hypoxia Is Not a Mechanism in FASD

In this study, we show that every bout of maternal ethanol consumption leads to a mild reduction in P_aO_2 at the end of infusion. However, this mild fall in maternal P_aO_2 did not result in a fall in fetal P_aO_2 showing that the fetus is well adapted to these mild decreases in maternal oxygen levels. Fetal differences from the adult are higher hemoglobin concentration, left-shifted oxygen-hemoglobin saturation curve, and higher cardiac output per unit body weight (Thornburg and Morton, 1994). Further, when we supplemented the ethanol-fed ewes with oxygen to abolish any mild decreases in the maternal P_aO_2, we observed no improvement in the cerebellar Purkinje cell number. The cerebellar Purkinje cell numbers were also not different when we mimicked fetal acidosis with or without the mild fall in maternal P_aO_2. This set of findings provide conclusive evidence that fetal hypoxia is not a mechanism in ethanol mediated fetal neuronal damage. In fact, the experimental evidence supporting ethanol-induced cerebral hypoxia as a mechanism of FASD is largely indirect. For example, both hypoxia and ethanol exposure during development induce similar craniofacial and neuronal deficits (Aitken and Schiff, 1986; Bronsky et al., 1986). Direct evidence that cerebral hypoxia may be a mechanism by which prenatal ethanol exposure causes FASDs was supplied by a report that an intravenous bolus of ethanol in fetal monkeys resulted in fetal hypoxemia (Mukherjee and Hodgen, 1982). However, the ethanol dose

and administration paradigm (intravenous bolus over two minutes) used in that study resulted in exceptionally high blood ethanol concentrations (BECs) of ~500 mg/dl. Additionally, that study was performed in anesthetized animals: anesthesia may have impeded the normal protective cardiovascular homeostatic responses to the actions of ethanol. Other studies have demonstrated that an acute or a chronic ethanol exposure during brain growth spurt does not result in fetal hypoxemia (Cudd et al., 2001b; Falconer, 1990; Patrick et al., 1985; Reynolds et al., 1996; Richardson et al., 1985; Smith et al., 1989a). In contrast, it has been demonstrated that chronic binge ethanol during the third-trimester-equivalent of human brain development leads to hypercapnea and acidosis-mediated increased cerebral blood flow leading to increased oxygen delivery (Parnell et al., 2007). This finding coupled with reports that fetal hemoglobin concentration is not altered in response to ethanol (Gleason and Hotchkiss, 1992; Richardson et al., 1985) provide convincing evidence that oxygen delivery to the brain is not compromised during the third trimester equivalent at these BECs. Further, studies have also demonstrated in rats during brain growth spurt that ethanol doses capable of reducing fetal cerebellar Purkinje cell number do not alter global brain intracellular high-energy phosphate concentrations (Cudd et al., 2000), an indication that brain oxygenation is not altered and that ethanol does not mediate hypoxic, anemic, ischemic or histotoxic hypoxia. Therefore, factors other than hypoxia are responsible for the fetal neurodevelopmental damage in response to moderate doses of ethanol exposure during the third trimester equivalent of human fetal development.

Ethanol-Induced Fetal Acidemia Is an Important Mechanism in Fetal Cerebellar Purkinje Cell Loss

Another finding in this study is that chronic binge ethanol mediated acidosis results in fetal cerebellar injury. When the pH_a pattern produced by ethanol was mimicked for 6 hours throughout the third trimester-equivalent of human brain development, it resulted in a reduction in fetal cerebellar Purkinje cell number. At least nine mechanisms have been proposed to explain the role of ethanol-induced acidemia in brain injury. First, acidosis results in alterations in amino acid homeostasis. We previously reported that an ethanol-induced fall in maternal pH_a by around 0.15 units results in a decrease in the concentrations of maternal glutamine and those amino acids that are synthesized from glutamine like arginine and citrulline, while the only amino acid that was elevated was glutamate (Ramadoss et al., 2007d) (Figure A-19). In support of these findings, others have reported that a decrease in pH by around 0.06 units, results in increased expression of renal glutamine system N transporter (SNAT 3), and increased renal extraction of glutamine (Karinch et al., 2007). Such decreases in amino acids during critical periods of fetal development may be very deleterious to fetal growth and development, and most significantly, several reports have suggested that decreases in glutamine leads to apoptosis and that glutamine supplementation prevents cell death by maintaining the levels of the anitoxidant glutathione, and by regulating signal transduction pathways for cellular proliferation and apoptosis (Mates et al., 2002). Second, both ethanol and acidosis induce increases in maternal and fetal glucocorticoid levels. Appropriate cortisol concentrations during gestation are essential for normal fetal

brain growth, and increases in cortisol during development could interfere with neuronal proliferation and differentiation (Bohn, 1984). Maternal ethanol consumption leading to a fall in maternal pH_a by around 0.15 units results in increased maternal and fetal ACTH and cortisol concentrations (Cudd et al., 2001a; Ramadoss et al., 2007c) (Figures A-27 and A-28), while acidemia (a fall in pH_a between 0.1 and 0.2 units), independent of ethanol has been demonstrated to increase ACTH and cortisol levels in adult dogs and in fetal sheep (Perez et al., 1979; Wiederkehr and Krapf, 2001; Wood and Chen, 1989). Third, both acidosis and ethanol enhances free radical production in brain homogenates as evidenced by increased lipid peroxidation (Ying et al., 1999) (Figures A-29 and A-30). Fourth, both ethanol and decreases in pH result in low serum IGF-1 concentrations. Maternal plasma IGF-1 concentrations were reduced by 51% in response to ethanol, with a 20% reduction in hepatic IGF-1 mRNA levels (Breese and Sonntag, 1995), and acidosis resulting from a fall in pH from 7.42 to 7.31 leads to a 36% reduction in plasma IGF-1 concentrations in adult non-pregnant humans (Brungger et al., 1997; Challa et al., 1993; Wiederkehr and Krapf, 2001). Fifth, cerebellar granule cell NMDA receptors are inhibited by a mild fall in extracellular pH with an EC_{50} of 7.3 (Traynelis et al., 1990; Traynelis et al., 1991). Therefore, a decrease in pH by around 0.15 units in response to alcohol may substantially affect the synaptic transmission to the Purkinje cells (Low et al., 2003). Moderate N-methyl D-Aspartate (NMDA) receptor (glutamate receptor) activation during development has trophic effects on the action of other neurotrophic factors (Langlais and Mair, 1990) and inhibition of these receptors may cause cell death. Sixth, acid sensitive ion channels called TASK (TWIK K^+) that are extremely sensitive

to variations in extracellular pH in a narrow physiological range (Duprat et al., 1997) has been identified in the cerebellar granule cells (Millar et al., 2000), and has been implicated in apoptosis in response to minute variations in extracellular pH (Lauritzen et al., 2003). Though it should be noted that this could not have been a mechanism of injury; actions of protons on these channels may only be potentially beneficial as they depolarize the granule cell leading to prevention of alcohol-induced decreases in NMDA receptor inhibition. Seventh, it has been suggested that there are ultrasensitive pH switches in proteins (for review, see Srivastava et al., 2007). In the cytosol, proteins have striking changes in activity over a few tenths of a pH unit and it has been suggested that structural changes induced by protons could promote phosphorylation, by making the site of phosphorylation accessible to a kinase (Srivastava et al., 2007). Eighth, we have demonstrated that respiratory acidosis similar to that induced by alcohol can have effects on multiple organ systems including renal function (Figure A-31). We found that the fractional clearance of chloride was increased with respect to sodium in response to acidosis. Finally, it has long been suggested that the threshold for acidemia or acidosis-induced death of neurons and glia is not a fixed value (Nedergaard et al., 1991). Rather, cell death is a combinatorial function of time and the degree of intracellular acidification (Nedergaard et al., 1991). Here, we propose that in addition to the duration and level of acidosis, the pattern of exposure may play a role in brain injury. Additional studies are required to understand how ethanol-induced acidosis mediates cell death, especially during brain growth spurt.

TASK Channel Inhibitor (TI) Prevents Prenatal Alcohol-Induced Purkinje Cell Death

The most important finding in this study is that inhibition of the novel tandem two pore domain acid sensitive potassium channel (TASK) completely prevents ethanol-induced Purkinje cell loss in the fetal cerebellum (Duprat et al., 1997). This protective effect could occur at least by two major mechanisms. First, TASK channels are abundantly expressed in the CNS, principally in the cerebellar granule cells (the excitatory interneurons that relay mossy fiber input to the Purkinje cells) (Millar et al., 2000; Plant et al., 2002). Cerebellar granule cells are one of the most populous cells in the mammalian brain and they express a standing outwardly rectifying potassium current (TASK current) which does not inactivate and is responsible for the large negative resting membrane potential in these cells (Watkins and Mathie, 1996; Han et al., 2002). They control the firing threshold and the firing frequency. They are insensitive to the classical broad spectrum potassium channel blocking drugs 4-aminopyridine and tetraethylammonium ions. It has also been reported that these channels cannot be considered as an 'M'-current or a delayed rectifier or a calcium activated current or a leak current (Watkins and Mathie, 1996). In this context, it should be noted that moderate N-methyl D-Aspartate (NMDA) receptor (glutamate receptor) activation during development has trophic effects on the action of other neurotrophic factors

(Langlais and Mair, 1990) and it is well known that developmental alcohol exposure induces arrest of cell differentiation through inhibition of NMDA receptor (Robbinson and Mair, 1992). Therefore, TI-induced depolarization of the cerebellar granule cells leading to increased excitability (Millar et al., 2002) and NMDA elicited calcium signals (Netzeband et al., 1999) may prevent ethanol-induced cell death. Second, TASK channels are abundantly present in the peripheral and central chemoreceptors and they control minute-minute ventilation in response to minute alterations in extracellular pH in adults. TIs act on these channels to increase the firing rate of action potentials in the afferent pathways leading to increased tidal volume and respiratory rate. This action of TI would directly prevent the fall in pH and thus prevent acidosis-induced nutrient and endocrine imbalance. In summary, this TASK channel blockade serves as a novel therapeutic target in treating FASD and may serve as an important means to prevent fetal neuronal loss in response to developmental alcohol exposure.

SUMMARY

Several important conclusions can be made from this study. First, the fetal cerebellum is an important target for ethanol teratogenesis. Second, maternal ethanol consumption does not lead to fetal hypoxia, and that hypoxia is not a mechanism involved in FASD. Third, prenatal ethanol exposure leads to significant hypercapnea and acidemia in the mother as well as in the fetus, and mimicking this pattern of acidosis produced by ethanol during the whole of the third trimester-equivalent partially accounts for the reduction in the fetal cerebellar Purkinje cell number. Finally, inhibiting the novel two pore domain acid-sensitive potassium channels expressed in the chemoreceptors and the cerebellar granule cells completely prevents the Purkinje cell loss elicited by alcohol. Future investigations will focus on interventions that involve a multimechanistic approach, and that are safe for use in pregnant women; we will inhibit pathways at the level of ethanol-induced acidosis (by increasing minute ventilation using a chemoreceptor stimulant) as well as downstream pathways that involve glutamine homeostasis, free radical injury, and glucocorticoid activity.

8. THREE TRIMESTER ALCOHOL EFFECTS ON THE BONES*

INTRODUCTION

Alcohol abuse during pregnancy can result in a variety of deleterious effects on the developing fetus including Fetal Alcohol Syndrome (Jones et al., 1973). In spite of significant efforts in educating women to not drink during pregnancy, the incidence of fetal alcohol effects has not decreased, making alcohol abuse during pregnancy an important health issue for women as well as the unborn child (Maternal and Child Health (MCH) data report, 2003; Institute of Medicine, 1996).

A cardinal feature of Fetal Alcohol Syndrome (FAS) is growth deficiency (Sokol and Clarren, 1989). Chronic heavy drinking during pregnancy is known to be deleterious to fetal bone development in both humans and laboratory animals. Growth deficits in children through 14 years of age have been associated with prenatal exposure to alcohol (Day et al., 2002). Chronic heavy drinking retards fetal skeletal development (Lee and Leichter, 1983; Keiver et al., 1996) and reduces ossification of fetal bones (Keiver et al., 1996) in rats. Even, moderate levels of developmental alcohol exposure have site-specific effects on the fetal skeleton (Simpson et al., 2005).

Although, previous studies have investigated the effects of developmental alcohol exposure on fetal skeletal ossification, no study has examined the effects of

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maternal alcohol binging on functional bone measurements. To date, there has been no study to compare the effects of moderate and heavy alcohol consumption on fetal and maternal bone strength. Largely for practical reasons, rats have been the most commonly used model to study the effects of prenatal alcohol exposure on the developing skeleton. However, a number of factors make the sheep arguably a better model for this purpose. The longer gestation in sheep (147 days) makes it easier to experimentally replicate human drinking patterns during pregnancy. The pattern of gestational development in sheep is more like that in the human; the rat is less mature at the time of parturition as demonstrated by the fact that the third trimester equivalent of brain growth occurs postnatally. And finally, the body mass of sheep is comparable to the human. Taken together, these differences make it easier to extrapolate bone strength and growth results between the sheep and the human. Our study is aimed at investigating, using a sheep model, the effects of moderate and high dose alcohol exposure throughout gestation on fetal and maternal bone strength and dimensions employing a weekly binge pattern, a drinking pattern common in women (Gladstone et al., 1996; Ebrahim et al., 1999; Maier and West, 2001a) who may have a greater likelihood of abstaining from drinking during pregnancy if convinced of it's importance.

METHODS

Animals and Breeding

The experimental procedures employed in this study were approved by the University Laboratory Animal Care Committee at Texas A&M University. Suffolk ewes (aged 2 to 6 years) maintained on coastal Bermuda grass pasture and supplemented with alfalfa hay were bred under controlled conditions. Time dated pregnancies were achieved by controlling the estrous cycle through the use of progesterone impregnated vaginal implants (EAZI-BREED™, CIDR®, Pharmacia & Upjohn Ltd., Auckland New Zealand). Implants were removed 11 days after placement at which time prostaglandin $F_{2\alpha}$ (LUTALYSE®, Pharmacia & Upjohn, Kalamazoo MI, 20 mg) was intramuscularly administered. The following day, ewes were placed with a ram fitted with a marking harness for a period of 24 hours. Marked ewes were assessed ultrasonographically on 25, 60 and 90 days to confirm pregnancy. On day 4 of gestation, the saline control and alcohol group subjects were moved into individual pens but were able to see herdmates in the adjacent pens at all times. Conditions of constant temperature (22 degree Celsius) and fixed light dark cycle (12:12) were maintained. Once confined, the saline and alcohol treatment group subjects received 2 kg/day of a complete ration (Sheep and Goat Pellet, Producers Cooperative Association, Bryan, TX). This meets the daily feed requirements of pregnant sheep as recommended by Nutrient Requirements of Sheep (1985) (American Sheep Industry Association Inc, 2002). Daily feed consumption was monitored; subjects in the alcohol and saline treatment groups consumed all of the food offered. Subjects in the normal control group remained in the pens with herdmates

throughout the study. Subjects in this group were offered, as a group, an equivalent amount of feed compared to subjects in the saline and alcohol control groups. However, individual feed consumption was not monitored.

Alcohol Dosing Protocol

Four groups, including two alcohol treatment groups: E075 (alcohol dosage of 0.75 g/kg body weight) and E175 (alcohol dosage of 1.75 g/kg body weight), a saline control group that received 0.9% saline of a volume of and at an infusion rate equivalent to that of the high alcohol dose, and a normal control group were studied. Infusion solutions were delivered intravenously by peristaltic pump (Masterflex, model 7014-20, Cole parmer, Niles IL) through a 0.2 µm bacteriostatic filter. Pumps were calibrated before infusion. Alcohol infusions were 40% W/V in sterile saline administered over one hour. Alcohol was dosed to mimic a common human binge pattern of drinking where ewes received alcohol on three consecutive days (Gladstone et al., 1996; Ebrahim et al., 1999; Cudd et al., 2001a; Maier and West, 2001a) followed by 4 days without alcohol beginning on day 4 of gestation.

Experiment Protocol

Subjects in the saline or alcohol group received infusions on three consecutive days per week followed by 4 days without infusion beginning on day 4 of gestation, a binge drinking paradigm. Our experiments began on day 4 and were terminated on day 133 of gestation. On the day of infusions, as on every other day, investigators entered

the room that housed the ewes at 0800 hr. Ewes were connected to the infusion pump by 0830 hr and alcohol was infused continuously over 1 hr, between 0830 and 0930 hr. On gestational day 4, an intravenous catheter (16 gauge, 5.25 in Angiocath^{TM} Becton Dickinson, Sandy, UT) was placed percutaneously into the jugular vein. On gestational day 42, after conception and pregnancy was confirmed ultrasonographically, the ewes underwent surgery to chronically implant femoral arterial and venous vascular access ports (V-A-P^{TM}, Model CP 47P, Access Technologies, Skokie IL). In brief, anesthesia was induced by administering diazepam (0.2 mg/kg intravenously, Abbott Laboratories, North Chicago, IL) and ketamine (4 mg/kg intravenously, Ketaset®, Fort Dodge, IA). The ewes were intubated and a surgical plane of anesthesia was maintained using isoflurane (0.5-2.5%, IsoFlo®, Abbott Laboratories, North Chicago, IL) and oxygen. Arterial and venous vascular access ports were placed subcutaneously in the flank region and the catheters were advanced into the aorta and vena cava via the femoral artery and vein respectively. At the end of surgery, the ewes received an injection of flunixin meglumine (1.1 mg/kg intramuscularly, Banamine®, Scherring-Plough, Union, NJ), a prostaglandin synthase inhibitor, to reduce postoperative pain. Ewes also received postoperative antibiotics (ampicillin trihydrate, polyflex®, Aveco, Fort Dodge, IA, 25 mg/kg administered subcutaneously for 5 days and gentamicin sulfate, Gentavet®, Velco, St.Louis, 2 mg/kg administered intramuscularly twice daily for 5 days). Infusions were then given through the venous port and blood was sampled from the arterial port.

Blood alcohol concentrations (BAC) were measured every 30 min for 2 hr and at 6 and 24 hrs beginning with the commencement of alcohol infusions on days 6, 40, 90 and 132. Blood was drawn from the jugular vein catheter on gestational days 6 and 40 and from femoral artery catheter on days 90 and 132 for the measurement of BAC. A 20 µl aliquot of blood was collected into microcapillary tubules and transferred into vials that contained 0.6 N perchloric acid and 4 mM n-propyl alcohol (internal standard) in distilled water. The vials were tightly capped with a septum sealed lid and were stored at room temperature until analysis by headspace gas chromatography (Varian Associates model 3400, Palo Alto, CA) at least 24 hr after collection. The basic gas chromatographic parameters were similar to those reported by Penton (1985), with the exception of the column (DB-wax, Megabore, J&W Scientific Folsum, CA) and the carrier gas (helium) used (Cudd et al., 2001b).

The fetuses were harvested on gestational day 133. The maternal and fetal femurs and tibias were collected, cleaned and stored at -20 degree celcius. Femur length was measured from the tip of the greater trochanter to tip of lateral condyle. Tibial length was measured from tip of the medial tubercle of the intercondyloid eminence to the tip of the medial malleoulus. Anterior-posterior as well as medio-lateral diameters were measured at mid-diaphyseal region using a precision caliper and the average diameter was calculated. Thawed bones were mechanically tested using a three-point bending procedure, which exclusively tests cortical bone in the mid-diaphysis region. For femur tests, the bones were placed on two lower supports centered with the anterior surface contacting the lower supports. For tibia tests, the lateral surface contacted the

lower supports. For fetal bones (femur and tibia), the distance between the lower supports was 4 cm, whereas for maternal bones (femur and tibia) this distance was 10 cm. Load was applied through the single upper contact that was centered between the two lower supports. The upper contact was advanced at a slow, quasi-static displacement rate of 5.08 mm/min (0.2 in/min) until complete fracture of the bone specimen occurred. Tests were conducted on an Instron (Norwood, MA) 1125 load frame with force measured by a load cell attached to the upper contact. The maximum force during the test was recorded for each bone. Displacements were not recorded. After the test, a thin cross-section was cut adjacent to the fracture location and a digital image was made of this cross-section. This image was analyzed to determine the cross-sectional moment of inertia (CSMI). Bone strength can be quantified in terms of both extrinsic, whole-bone, structural properties, such as maximum force, and in terms of intrinsic, tissue-level, material properties. A simple way to estimate intrinsic properties is to normalize the maximum force to body weight. A more detailed and comprehensive approach is to determine the material-level stress using appropriate engineering mechanics calculations. In this study, the stress corresponding to the maximum force is

referred to as bone "strength" and was calculated by classical beam theory analysis using the following equation:

$$S = F L d / (8I)$$

where S is the bone strength (MPa), F is the maximum force (N), L is the distance between the lower supports (40mm or 100mm), d is the mid-diaphysis diameter (mm), and I is the cross-sectional moment of inertia (mm^4).

Data Analysis

Data are presented as the mean ± SEM. Group differences were determined by one-way ANOVA followed by Student Newman-Keuls test. Statistical significance was established *a priori* at $p<0.05$ while p values between 0.05 and 0.10 were considered trends. A two-way ANOVA for the factors time interval and the day of gestation was performed to compare BACs at different times during gestation. Maternal body weights were analyzed by two-way ANOVA for the factors group and day of gestation.

RESULTS

A total of 10 ewes were assigned to each group. Animals were lost from the study as a result of fetal, but not maternal demise. The incidence of fetal demise was similar among all treatment groups (Final numbers; normal control group, n = 8; saline control group, n = 7; E075 group, n = 8; E175 group, n = 6) suggesting that losses were unrelated to the alcohol treatment.

Blood Alcohol Concentration

The mean BACs measured on days 6, 41, 90 and 132 of gestation for E075 and E175 groups peaked at 1 hr which coincided with the end of the infusion period. The blood alcohol concentrations on the different days for either E075 or E175 group did not differ significantly (two-way ANOVA, E075 group, $p = 0.695$; two-way ANOVA, E175 group, $p = 0.477$) and were therefore combined (Figure A-32).

Growth

Fetal body weights (normal control group, 4.7 ± 0.2 kg; saline control group, 4.3 ± 0.3 kg; E075 group, 4.7 ± 0.2 kg; E175 group, 4.6 ± 0.4 kg) and lengths (normal control group, 50.4 ± 0.7 cm; saline control group, 50.1 ± 1.2 cm; E075 group, 50.0 ± 1.4 cm; E175 group, 49.5 ± 2.1 cm) were not different among groups (one-way ANOVA, fetal body weight, $p = 0.647$; one-way ANOVA, length, $p = 0.973$). Fetal femoral lengths significantly differed among groups (one-way ANOVA, $p = 0.042$). Post-hoc tests indicated that fetal femurs in the E175 group were shorter than in the

normal control group ($p = 0.039$) while the E075 group showed a trend towards shorter lengths ($p = 0.079$) (Figure A-33). However, the fetal femoral lengths in the alcohol exposed groups were not significantly different from the saline control group. Mean fetal tibial lengths did not exhibit dose-dependent changes (Figure A-34). The differences among treatment groups approached significance (one-way ANOVA, $p = 0.057$). However, the alcohol groups, when combined, had shorter fetal tibias (one-way ANOVA, $p = 0.030$). The mean fetal tibial lengths were 11.7 ± 0.2 cm, 11.8 ± 0.2 cm, 10.7 ± 0.3 cm for normal control group, saline control group and the combined alcohol groups respectively. The fetal tibias in the alcohol exposed fetuses showed significantly shorter mean lengths compared to the normal control group ($p = 0.031$) and a strong trend toward shorter lengths compared to the saline control group ($p = 0.067$).

Neither fetal nor maternal mean diameter or cross sectional moments of inertia for both femur and tibia exhibited any significant change compared to the control groups at either doses (Tables B-1 and B-2 respectively). Maternal body weights measured at the beginning of the study (gestational day 4) were not different between groups and did not change significantly at gestational day 42. Maternal body weights were progressively higher at day 70, 90 and 132 in the individuals where weights were recorded (3 in the E075 group and 1 in the E175 group). Ewes in the normal control group were not weighed. All ewes were judged to be of normal body condition at the time of sacrifice.

Bone Strength

For all of the fetal bones, there were no significant differences among all of the groups for the maximum force, which is an extrinsic property and indicative of the contributions of both tissue mineralization and bone size and shape. Upon normalization by body weight, however, the maximum force per unit fetal body weight showed a strong trend toward differences (femur, one-way ANOVA, $p = 0.065$; tibia, one-way ANOVA, $p = 0.065$), with similar patterns for the fetal femur (normal control group, 183.6 ± 10.6 N/kg; saline control group, 171.6 ± 9.3 N/kg; E075 group, 193.6 ± 13.3 N/kg; E175 group, 135.9 ± 24.8 N/kg) and fetal tibia (normal control group, 204.6 ± 13.1 N/kg; saline control group, 196.0 ± 15.1 N/kg; E075 group, 209.1 ± 14.5 N/kg; E175 group, 148.9 ± 24.0 N/kg).

The more detailed and comprehensive determination of intrinsic tissue-level strengths from beam bending theory indicated that prenatal binge alcohol exposure resulted in significant differences in fetal femoral strength (one-way ANOVA, $p=0.004$) (Figure A-35). The higher dose (E175) group had significantly lower femoral strength compared to the lower dose (E075) group ($p = 0.006$) and strong trend towards lower strengths compared to the pair-fed saline control group ($p = 0.089$). The lower dose (E075) group showed significantly higher femoral strength compared to both the normal

control group ($p = 0.008$) and the higher dose alcohol (E175) group ($p = 0.006$). However, the lower dose (E075) group did not exhibit higher femoral strength compared to the saline control group ($p = 0.15$).

The mean fetal tibial strength was greatest for the normal control group (one-way ANOVA, $p = 0.016$) (Figure A-36). The lower dose group (E075) did not cause any deleterious effect on the tibial strength but the higher dose group (E175) was decidedly lower in strength. Fetal tibial strength of the E175 group was significantly lower compared to both the normal control group ($p = 0.019$) and the E075 group ($p = 0.082$) but was not different compared to the saline control group. However, the saline control group trended to have a lower strength compared to the normal control group ($p = 0.056$).

Binge alcohol exposure throughout gestation did not produce statistically significant differences on the maternal bones (Tables B-1-3).

DISCUSSION

Binge consumption of alcohol throughout gestation produced alterations in fetal, but not maternal bone. The most important finding in this study is that the higher alcohol dose resulted in reduced fetal femoral strength. Tibial strengths were also lower in the higher alcohol dose group when compared with the normal control group and the lower alcohol dose group. In contrast, the lower alcohol dose increased fetal femoral strength compared to the normal control subjects. It should be recalled that there were no differences in extrinsic, whole-bone properties (maximum force), and that "strength" in this context refers to the intrinsic, tissue-level stress associated with the maximum force. It is also worth noting that simply normalizing maximum force to body weight revealed trends similar to the bone strength (stress) results, which tends to confirm the findings of material-level effects despite no whole-bone effects. Alcohol exposed fetuses had shorter femurs compared to the normal control group but the tibia exhibited shorter lengths only when the alcohol groups were combined. However, the effects of alcohol on the bone were independent of the effects on overall growth (as measured by body weight and length) as the alcohol exposed fetuses were not smaller than the control fetuses. This is consistent with findings in a recent study (Simpson et al., 2005) where moderate levels of maternal alcohol consumption had effects on fetal skeletal development independent of its effects on overall fetal body weight and length.

The finding that the tibial strength in the higher dose group was different from the normal control group but not from the pair-fed saline control group could suggest that reduced tibial strengths are attributable to instrumentation and nutrition. However,

it should be noted that the lower dose group which was instrumented and also received a feed equivalent to the saline control group trended to exhibit higher tibial strength compared to the higher dose group. Thus, it is difficult to interpret whether reduced tibial strength was due to alcohol versus nutrition and instrumentation. Further, it should also be noted that the failure to reach significance may have been due to the relatively low statistical power of this experiment. Further, the normal control subjects in this study were fed as a group and thus, individuals in this group may have received an amount of feed different from the treatment groups while the saline subjects received a daily amount of feed equivalent to the alcohol treatment groups. The higher dose group subjects also displayed shorter femurs but only when compared to the normal control group subjects. Fetal tibias trended to be different among treatment groups but fetal alcohol exposure resulted in lower lengths only when the two alcohol groups were combined.

Developmental alcohol exposure is complicated by the existence of multiple mechanisms by which alcohol mediates damage, depending upon the dose and the pattern of exposure and the timing of exposure relative to development (Cudd, 2005). Pre- (Lee and Leichter, 1983; Keiver et al., 1996, Keiver et al, 1997) and postnatal (Lee and Leichter, 1980) growth deficits in rats are associated with alcohol exposure *in utero*. Though human studies show the effects of alcohol on body length (Day et al., 1989; Jacobson et al., 1994; Day et al., 2002), it should be noted that human studies depend on unreliable self-reporting, and most heavy drinkers also use tobacco or other drugs that alone or together with alcohol may interfere with fetal development. Further, nutrition is

also difficult to control in human studies (Cudd, 2005). Maternal alcohol consumption has been shown to reduce fetal skeletal ossification (Lee and Leichter, 1983; Keiver et al., 1996, Keiver et al, 1997; Keiver and Weinberg, 2004). Though, a number of studies have reported the effects of prenatal alcohol exposure on fetal skeletal ossification, no previous study had examined the effects on functional measures that include the strength of the bone. This is the first study to examine the effects of maternal alcohol consumption during all three trimesters on fetal and maternal bone strength.

The three point bending procedure tests primarily cortical, rather than trabecular, bone. The decreased cortical bone strength in the higher alcohol dose group may have important consequences as a decrease in cortical envelope may have a persistent effect on trajectory of bone growth later in childhood (Tobias et al., 2004). There are numerous potential mechanisms by which alcohol might affect fetal bone development and strength, including direct and indirect effects on placental function, bone/cartilage cell function, differentiation etc. It has been reported that prenatal alcohol exposure impairs calcium homeostasis, possibly by altering maternal serum levels of vitamin D (Keiver et al., 1996). It has been proposed that alcohol influences fetal bone development through actions on parathyroid hormone-related peptide (PTHrp) (Keiver et al., 1997; Keiver and Weinberg, 2004). PTHrp gene depleted mice show a defect in endochondral bone formation (Amizuka et al., 1994). The ovine fetal and maternal parathyroid gland and the placental extracts show the presence of PTHrp (Rodda et al., 1988) but the effects of prenatal alcohol exposure on ovine fetal PTHrp have not been investigated.

In contrast, the lower dose group showed significantly higher femoral strength compared to both the normal control group and the higher dose group but not the saline control group. Even though, the increased strength was not relative to the saline control group, it is nevertheless an interesting observation. This suggestion, that a low alcohol dose may enhance aspects of fetal bone development, is not without precedent as another study has found that moderate alcohol consumption increased bone chemistry and histomorphometric values for both tibia and femur in rats (Sampson et al., 1999). Mechanisms that involve alcohol mediated increases in estrogen, nitric oxide or IGF II could be attributed to the higher fetal bone strength in the lower dose group as all of these have been demonstrated to have a positive influence on skeletal development (Migliaccio et al., 1996; Aguirre et al., 2001; McCarthy et al., 1989). Enhanced estrogen exposure during the prenatal period increases bone mass in mice (Migliaccio et al., 1996). Elevated levels of estrogen enhance the anabolic actions of parathyroid hormone on bone (Xiao-yi et al., 1994). Alcohol increases circulating estrogen levels by increasing aromatization of androgen to estrogen (Chung, 1990; Turner and Sibonga, 2001). It is also hypothesized that alcohol may also increase circulating estrogen levels by decreasing the metabolism of estrogen (Turner and Sibonga, 2001). Nitric oxide has recently been found to have important effects on bone (Evans et al., 1996) with endothelial NOS gene-deficient mice having marked abnormalities including significant retardation in bone formation (Aguirre et al., 2001). Moderate alcohol consumption increases the expression of endothelial NOS (Venkov et al., 1999) and binge drinking causes a rise of serum nitric oxide concentration (Oekonomaki et al., 2004). In addition,

estrogen treatment increases the endothelial constitutive NOS expression in human osteoblast-like cells (Armour et al., 1998). Further, there is evidence that IGF II may also have a role in influencing prenatal growth as plasma IGF II levels are higher in the fetus than in the adults (Gluckman and Butler, 1983). Plasma IGF II concentration was found to be increased nearly by 100% in alcohol fed pregnant rats (Breese and Sonntag, 1995) and this may be responsible for the increased bone strength observed in our study as alteration of plasma IGF regulation may contribute to changes in maternal and placental metabolism and hormone regulation during pregnancy (Breese and Sonntag, 1995). Therefore it is possible that the potential protective effects of estrogen, nitric oxide and IGF II may be important mechanisms by which moderate maternal alcohol consumption results in higher fetal bone strength.

We did not observe significant differences in bone strength in the mother at either dose. Previous studies showed that alcohol-exposed, young, actively growing rats had lower bone strengths (Hogan et al., 1997), but mechanical properties of the femoral diaphysis were largely unaffected in alcohol exposed adult rats (Hogan et al., 2001). The three point bending procedure tests only the cortical bone whose primary function is strength and support in contrast to cancellous bone that functions more prominently in metabolic activities with a combined mechanical role of energy absorption. Previous studies have demonstrated that the mechanical properties of the cancellous bone in alcohol-fed adult rats were significantly diminished in the distal femur but alcohol-exposed cortical bones were largely unaffected (Hogan et al., 2001) as was the case in the present study. This negative finding also may be due to the relatively low peak

blood alcohol concentrations (189.02 $\pm$ 16.85 mg/dl) and because of the relatively restricted pattern of exposure (3 consecutive binges per week over the length of the gestation).

Pre- and postnatal growth deficiency is a cardinal feature of FAS, but little is known about the mechanisms responsible for such deficits. Our investigation demonstrated that binge alcohol exposure throughout gestation can influence fetal bone development. There is a strong need for further research to determine the specific mechanisms by which alcohol mediates the changes in fetal skeletal development. The effects of alcohol were site-specific which is consistent with findings by others where prenatal alcohol exposure affected some bones more than the others (Simpson et al., 2005). Because the effects of alcohol on fetal bone strength are dose-dependent and site specific, likely the actions of alcohol on bone involve multiple mechanisms.

9. FIRST TRIMESTER ALCOHOL EFFECTS ON THE BONES

INTRODUCTION

A cardinal feature of Fetal Alcohol Syndrome (FAS) is growth retardation (Jones et al., 1973). Prenatal alcohol exposure has been demonstrated to affect size at age 14 years in children followed since their fourth month of gestation (Day et al., 2002). Decreased whole body weight and length are observed in human and animal model systems (Day et al., 2002; Jones et al., 1973; Keiver and Weinberg, 2004). In addition to the adverse outcomes on the whole body growth parameters, chronic heavy drinking during pregnancy is known to be deleterious to fetal bone development in both humans and animals. Prenatal alcohol exposure has been shown to impair skeletal development through impairment of calcium homeostasis (Keiver and Weinberg, 2004) regardless of the duration of consumption (Keiver and Weinberg, 2003) in rats. These impairments result in retarded prenatal and postnatal skeletal development (Lee and Leichter, 1979, 1983). We previously reported that all three trimester binge alcohol exposure results in reduced fetal bone strength at higher doses and enhanced bone strength at lower doses in a sheep model. The large body mass of the ovine fetus, the longer gestation that is more similar to that of humans, and that all three trimester equivalents occur *in utero*, make the sheep a good model for studying the effects of maternal alcohol consumption on the fetal skeleton. In this study, we wished to examine the effects of first trimester moderate and heavy alcohol binging on the mechanical properties of the bone.

METHODS

Animals and Breeding

The experimental procedures employed in this study were approved by the University Laboratory Animal Care Committee at Texas A&M University. Suffolk ewes (aged 2 to 6 years) maintained on coastal Bermuda grass pasture and supplemented with alfalfa hay were bred under controlled conditions. Time dated pregnancies were achieved by controlling the estrous cycle through the use of progesterone impregnated vaginal implants (EAZI-BREED™, CIDR®, Pharmacia & Upjohn Ltd., Auckland New Zealand). Implants were removed 11 days after placement at which time prostaglandin $F_{2\alpha}$ (LUTALYSE®, Pharmacia & Upjohn, Kalamazoo MI, 20 mg) was intramuscularly administered. The following day, ewes were placed with a ram fitted with a marking harness for a period of 24 hours. Marked ewes were assessed ultrasonographically on 25, 60 and 90 days to confirm pregnancy. On day 4 of gestation, the saline control and alcohol group subjects were moved into individual pens but were able to see herdmates in the adjacent pens at all times. Conditions of constant temperature (22 degree Celsius) and fixed light dark cycle (12:12) were maintained. Once confined, the saline and alcohol treatment group subjects received 2 kg/day of a complete ration (Sheep and Goat Pellet, Producers Cooperative Association, Bryan, TX).

Experiment Protocol

Three groups, including two alcohol treatment groups: E075 (alcohol dosage of 0.75 g/kg body weight) and E175 (alcohol dosage of 1.75 g/kg body weight), and a

saline control group that received 0.9% saline of a volume of and at an infusion rate equivalent to that of the high alcohol dose were studied. Infusion solutions were delivered intravenously by peristaltic pump (Masterflex, model 7014-20, Cole parmer, Niles IL) through a 0.2 µm bacteriostatic filter. Pumps were calibrated before infusion. Alcohol infusions were 40% W/V in sterile saline administered over one hour. Alcohol was dosed to mimic a common human binge pattern of drinking where ewes received alcohol on three consecutive days (Gladstone et al., 1996; Ebrahim et al., 1999; Cudd et al., 2001a; Maier and West, 2001a) followed by 4 days without alcohol beginning on day 4 of gestation till day 41 of gestation. On GD 133, the ewes were euthanized using sodium pentobarbital (75 mg/kg, intravenously), and the fetuses were removed from the uterus and perfused with saline followed by cold fixative solution containing 1.25% paraformaldehyde and 3% glutaraldehyde in phosphate buffer (pH, 7.4).

Bone Testing

The maternal and fetal femurs and tibias were collected, cleaned and stored at -20 degree celcius. Femur length was measured from the tip of the greater trochanter to tip of lateral condyle. Tibial length was measured from tip of the medial tubercle of the intercondyloid eminence to the tip of the medial malleoulus. Anterior-posterior as well as medio-lateral diameters were measured at mid-diaphyseal region using a precision caliper and the average diameter was calculated. Thawed bones were mechanically tested using a three-point bending procedure, which exclusively tests cortical bone in the mid-diaphysis region. For femur tests, the bones were placed on two lower supports

centered with the anterior surface contacting the lower supports. For tibia tests, the lateral surface contacted the lower supports. For fetal bones (femur and tibia), the distance between the lower supports was 4 cm, whereas for maternal bones (femur and tibia) this distance was 10 cm. Load was applied through the single upper contact that was centered between the two lower supports. The upper contact was advanced at a slow, quasi-static displacement rate of 5.08 mm/min (0.2 in/min) until complete fracture of the bone specimen occurred. Tests were conducted on an Instron (Norwood, MA) 1125 load frame with force measured by a load cell attached to the upper contact. The maximum force and the displacement during the test were recorded for each bone. After the test, a thin cross-section was cut adjacent to the fracture location and a digital image was made of this cross-section. This image was analyzed to determine the cross-sectional moment of inertia (CSMI). Bone strength can be quantified in terms of both extrinsic, whole-bone, structural properties and in terms of intrinsic, tissue-level, material properties. In this study, we examined the following extrinsic and intrinsic properties: yield force (N), stiffness (N/mm), ultimate load (N), energy-to-ultimate (mJ), and bone strength (MPa). In this study, the stress corresponding to the maximum force

is referred to as bone "strength" and was calculated by classical beam theory analysis using the following equation:

$$S = F L d / (8I)$$

where S is the bone strength (MPa), F is the maximum force (N), L is the distance between the lower supports (40mm or 100mm), d is the mid-diaphysis diameter (mm), and I is the cross-sectional moment of inertia (mm^4). A representative sample of a tibial test is shown in figure A-37.

Data Analysis

Femur and tibia bone properties were analyzed using a one-way ANOVA with treatment group as between factor. Post-hoc was performed using Student Newman-Keuls test. Statistical significance was established *a priori* at $p<0.05$. Data are presented as the mean ± SEM.

RESULTS

A total of 22 ewes were assigned to each group. The incidence of fetal demise was similar among all treatment groups (Final numbers; saline control group, n = 20; E075 group, n = 16; E175 group, n = 17) suggesting that losses were unrelated to the alcohol treatment. The mean peak BAC was 210 mg/dl.

Growth Parameters

There were no differences in the length and weight for any of the fetal bones among groups.

Extrinsic Bone Properties

For all of the fetal bones, there were no significant differences among the groups for the yield force (Figure A-38), stiffness (Figure A-39), and maximum force (Figure

A-40). However, the values for the energy to ultimate (Figure A-41) was different among groups ($F_{2,49} = 3.77$, $p = 0.03$). Post hoc tests showed that the lower alcohol dose was significantly higher compared with the control ($p = 0.009$). This is indicative of the role of a lower dose alcohol in both tissue mineralization and bone size and shape. Further, no differences were detected in any of the mechanical properties for the fetal tibia.

Intrinsic Properties

The more detailed and comprehensive determination of intrinsic tissue-level strengths from beam bending theory indicated that prenatal binge alcohol exposure resulted in significant differences in fetal femoral strength ($F_{2,43} = 3.404,$, $p=0.014$) (Figure A-42). The higher dose group had a significantly stronger bone strength compared to the control ($p = 0.004$). No differences were detected in tibia among groups.

DISCUSSION

This is the first study to our knowledge that has examined the functional properties of the bone in response to alcohol restricted to the first trimester of gestation in a model where all three trimester-equivalents occur *in utero*. The most important finding in this study is that the higher alcohol dose resulted in increased fetal femoral strength, and the lower dose group resulted in increased values for the energy to ultimate. Tibial properties were not altered among groups, showing site specific effects of alcohol.

This observation that alcohol leads to enhanced mechanical properties is not without precedent as another study has found that moderate alcohol consumption increased bone chemistry and histomorphometric values for both tibia and femur in rats (Sampson et al., 1999). Mechanisms that involve alcohol mediated increases in estrogen, nitric oxide or IGF II could be attributed to the higher fetal bone strength in the lower dose group as all of these have been demonstrated to have a positive influence on skeletal development (Migliaccio et al., 1996; Aguirre et al., 2001; McCarthy et al., 1989). Enhanced estrogen exposure during the prenatal period increases bone mass in mice (Migliaccio et al., 1996). Elevated levels of estrogen enhance the anabolic actions of parathyroid hormone on bone (Xiao-yi et al., 1994). Alcohol increases circulating estrogen levels by increasing aromatization of androgen to estrogen (Chung, 1990; Turner and Sibonga, 2001). It is also hypothesized that alcohol may also increase circulating estrogen levels by decreasing the metabolism of estrogen (Turner and Sibonga, 2001). Nitric oxide has recently been found to have important effects on bone

(Evans et al., 1996) with endothelial NOS gene-deficient mice having marked abnormalities including significant retardation in bone formation (Aguirre et al., 2001). Moderate alcohol consumption increases the expression of endothelial NOS (Venkov et al., 1999) and binge drinking causes a rise of serum nitric oxide concentration (Oekonomaki et al., 2004). In addition, estrogen treatment increases the endothelial constitutive NOS expression in human osteoblast-like cells (Armour et al., 1998). Further, there is evidence that IGF II may also have a role in influencing prenatal growth as plasma IGF II levels are higher in the fetus than in the adults (Gluckman and Butler, 1983). Plasma IGF II concentration was found to be increased nearly by 100% in alcohol fed pregnant rats (Breese and Sonntag, 1995) and this may be responsible for the increased bone strength observed in our study as alteration of plasma IGF regulation may contribute to changes in maternal and placental metabolism and hormone regulation

during pregnancy (Breese and Sonntag, 1995). Therefore it is possible that the potential protective effects of estrogen, nitric oxide and IGF II may be important mechanisms by which moderate maternal alcohol consumption results in higher fetal bone strength.

Pre- and postnatal growth deficiency is a cardinal feature of FAS, but little is known about the mechanisms responsible for such deficits. Our investigation demonstrated that binge alcohol exposure throughout gestation can influence fetal bone development. There is a strong need for further research to determine the specific mechanisms by which alcohol mediates the changes in fetal skeletal development. The effects of alcohol were site-specific which is consistent with findings by others where prenatal alcohol exposure affected some bones more than the others (Simpson et al., 2005). Because the effects of alcohol on fetal bone strength are dose-dependent and site specific, likely the actions of alcohol on bone involve multiple mechanisms.

10. SUMMARY AND CONCLUSIONS

In summary, these set of experiments establish the ovine model system as an excellent model for FASD research; alcohol administration to pregnant ewes can produce deleterious effects in most of the same brain regions and organ systems that have been reported in humans with FASD. These studies also establish the similarities in the temporal vulnerability of neuronal populations between humans and sheep; the brain growth spurt occurs in utero in sheep as in humans and the role played by the mother and the placenta are taken into consideration. The experiments also answer some key questions regarding the mechanisms underlying FASD involving maternal-fetal interactions in the ovine model system.

We conclude that chronic alcohol does not lead to hypoxic, anemic, ischemic or histotoxic cerebral hypoxia. Instead, it leads to increased oxygen delivery, increased cerebral blood flow specific to the fetal cerebellum, hypercapnea, acidemia, and fetal cerebellar Purkinje cell loss. Alcohol-induced hypercapnea and acidemia further result in a cascade of events in the maternal and fetal compartments that include deficits in the levels of glutamine and glutamine-related amino acids, alterations in endocrine axes, oxidative stress, alteration in cardiovascular homeostasis and fetal neuronal loss. Finally, the experiments demonstrate and/or suggest potential therapeutic strategies that involve the two pore domain acid sensitive channels and glutamine-related pathways to prevent or mitigate some of the devastating effects of chronic prenatal alcohol exposure on the fetal brain.

Therefore, these findings may be useful to develop intervention/amelioration strategies, a goal that is consistent with the NIAAA FY 08-12 Strategic Plan that encourages researchers to "Use knowledge gained in uncovering target sites for alcohol's action on the embryonic and fetal stages of life to begin developing potential therapeutic or preventative interventions, including dietary supplements (e.g., antioxidants and choline) that are safe for use in pregnant women". We also predict that a more effective intervention will likely require a combination of compounds that address the multimechanistic alcohol damage through more than one approach. The findings from these set of experiments will place us in an excellent position to understand the multimechanistic causes of alcohol damage and therefore to correctly predict and propose in the future a combined therapy that will have real promise as a preventative strategy.

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APPENDIX A

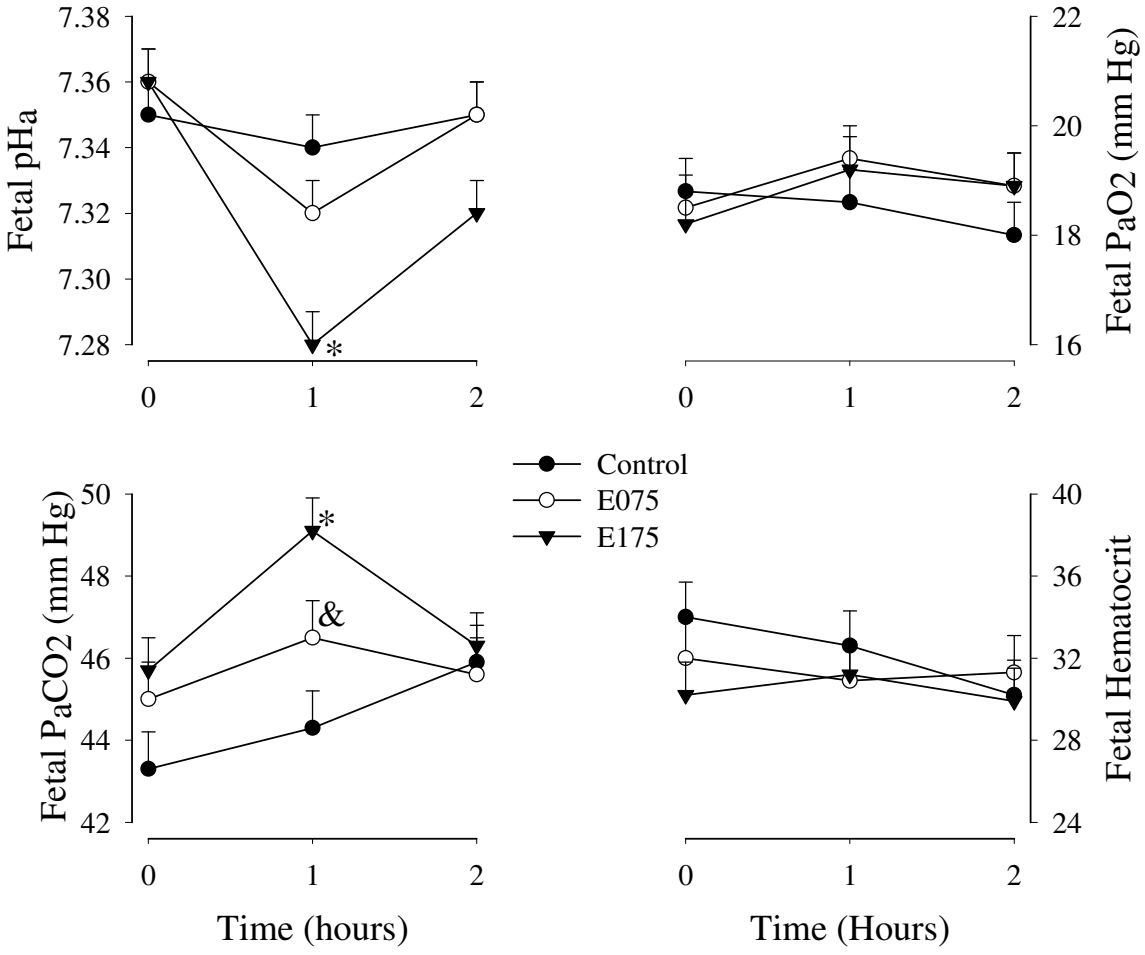

Figure A-1: Fetal arterial blood gases and hematocrit. Arterial pH (pH_a) was significantly (*) lower and partial pressure of carbon dioxide (P_aCO_2) was significantly higher at one hour in the E175 group (received 1.75 g/kg ethanol over one hour) compared to the control and the E075 groups (received 0.75 g/kg ethanol over one hour). P_aCO_2 was significantly elevated (&) in the E075 group also at the peak hour compared with that in control. The hematocrit and arterial partial pressure of oxygen (P_aO_2) were not different among groups.

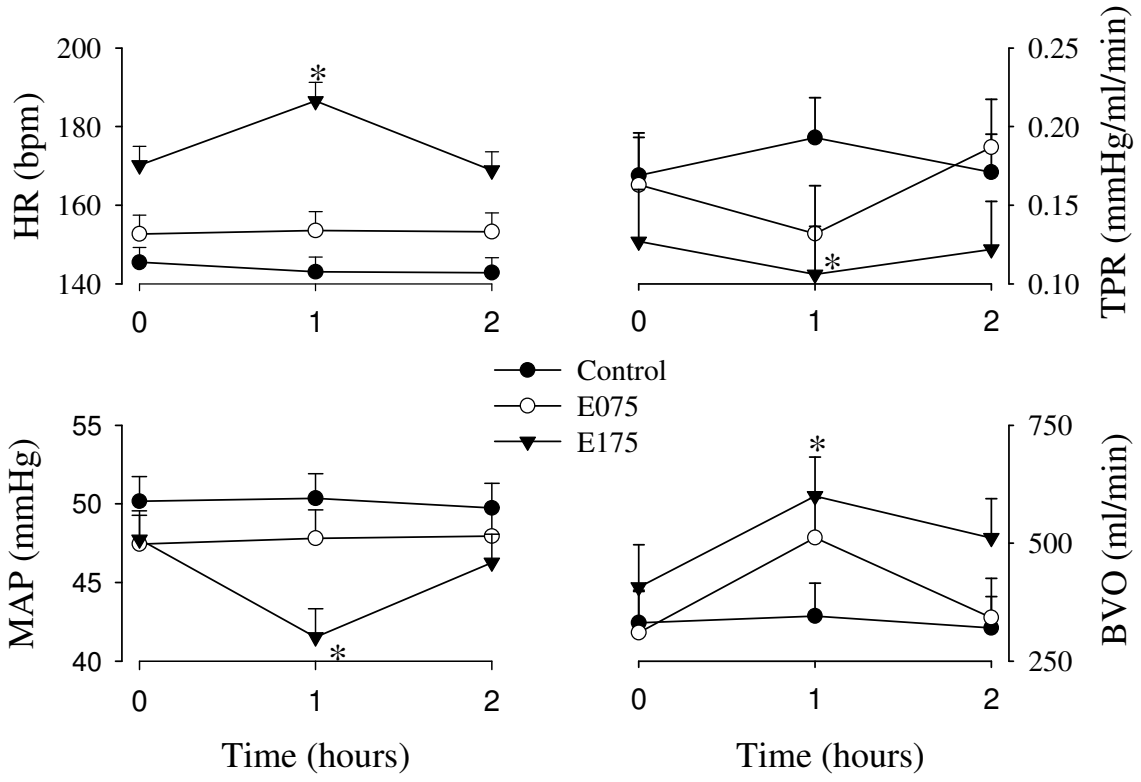

Figure A-2: Fetal hemodynamic variables. The E175 group (received 1.75 g/kg ethanol over one hour) exhibited significantly elevated (*) heart rate, decreased mean arterial pressure, elevated biventricular output and decreased total peripheral resistance at one hour compared to the control group. The E075 group (received 0.75 g/kg ethanol over one hour) was not different from the control group.

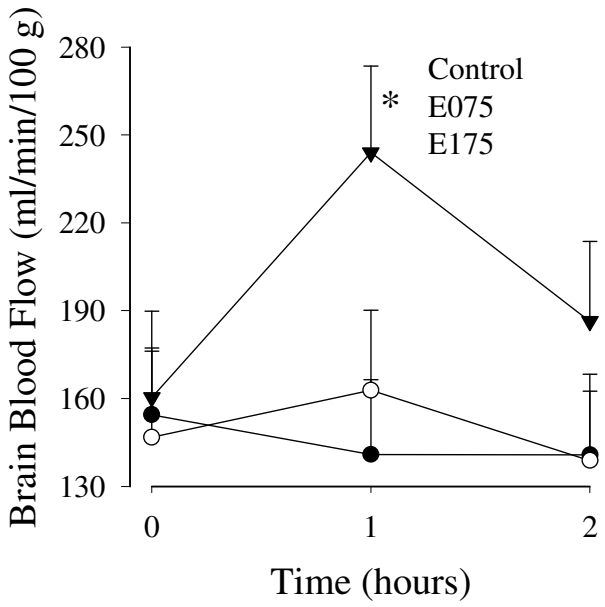

Figure A-3: Fetal whole brain blood flow (ml/min/100 g tissue). The E175 group (received 1.75 g/kg ethanol over one hour) exhibited significantly elevated (*) whole brain blood flow at one hour compared to the control group. The E075 group (received 0.75 g/kg ethanol over one hour) was not different from the control group.

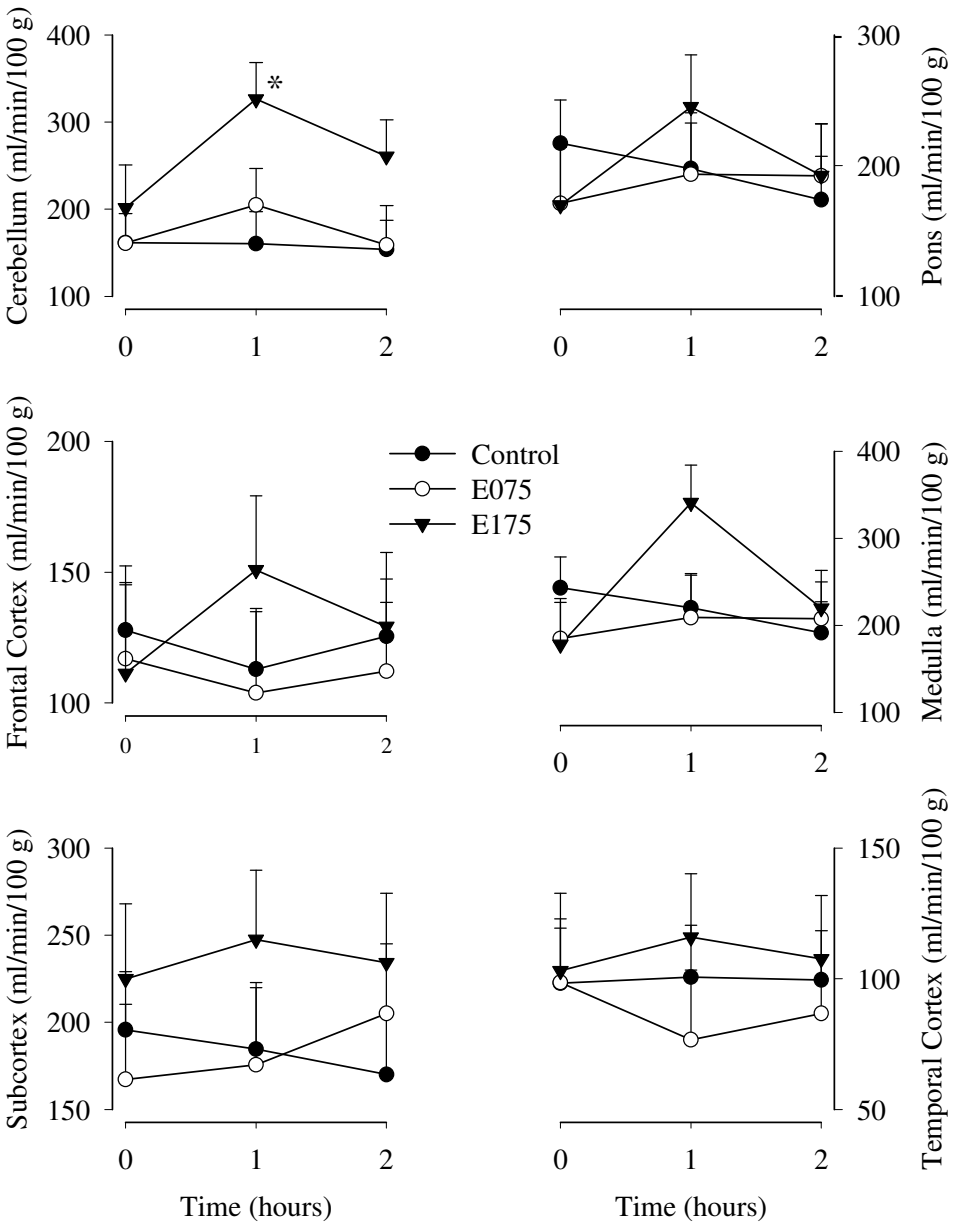

Figure A-4: Fetal regional brain blood flow (ml/min/100 g tissue). Blood flow in the E175 group (received 1.75 g/kg ethanol over one hour) was significantly elevated (*) only in the cerebellum at one hour compared to the control group. The values in pons, medulla, subcortex, frontal cortex, and temporal cortex trended numerically upwards at one hour in the E175 group, but the effect did not reach statistical significance.

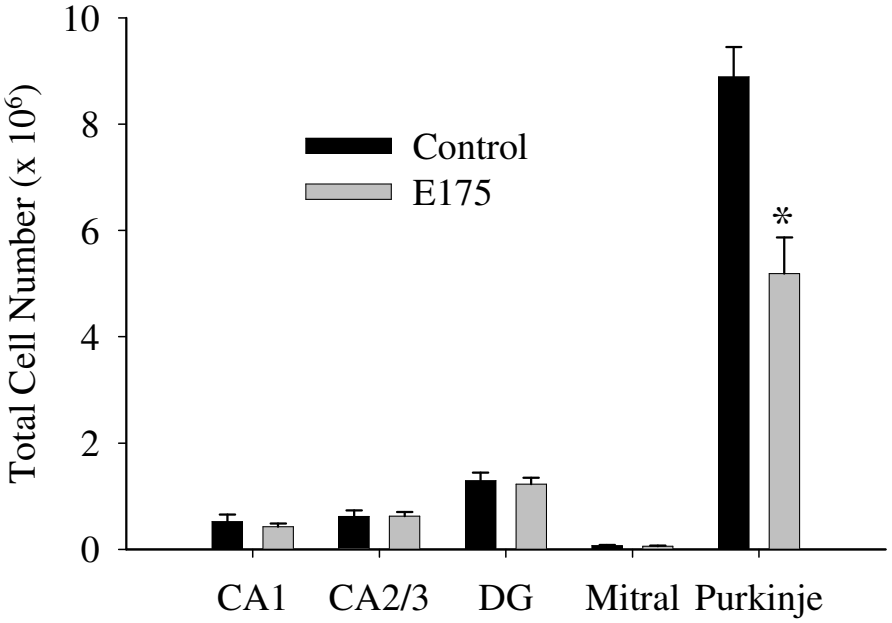

Figure A-5: Regional vulnerability in response to chronic binge ethanol. The E175 group (received 1.75 g/kg ethanol over one hour) displayed significantly lower (*) number of cerebellar Purkinje cells compared with that in the control group. However, no such differences were observed in other brain regions like the hippocampus CA1 and CA2/3 pyramidal cells, dentate gyrus granule cells (DG) or the olfactory bulb (mitral cells). These data demonstrate the extereme regional differences of the teratogenic effects of ethanol and the strong correlation between regional blood flow and neuronal loss.

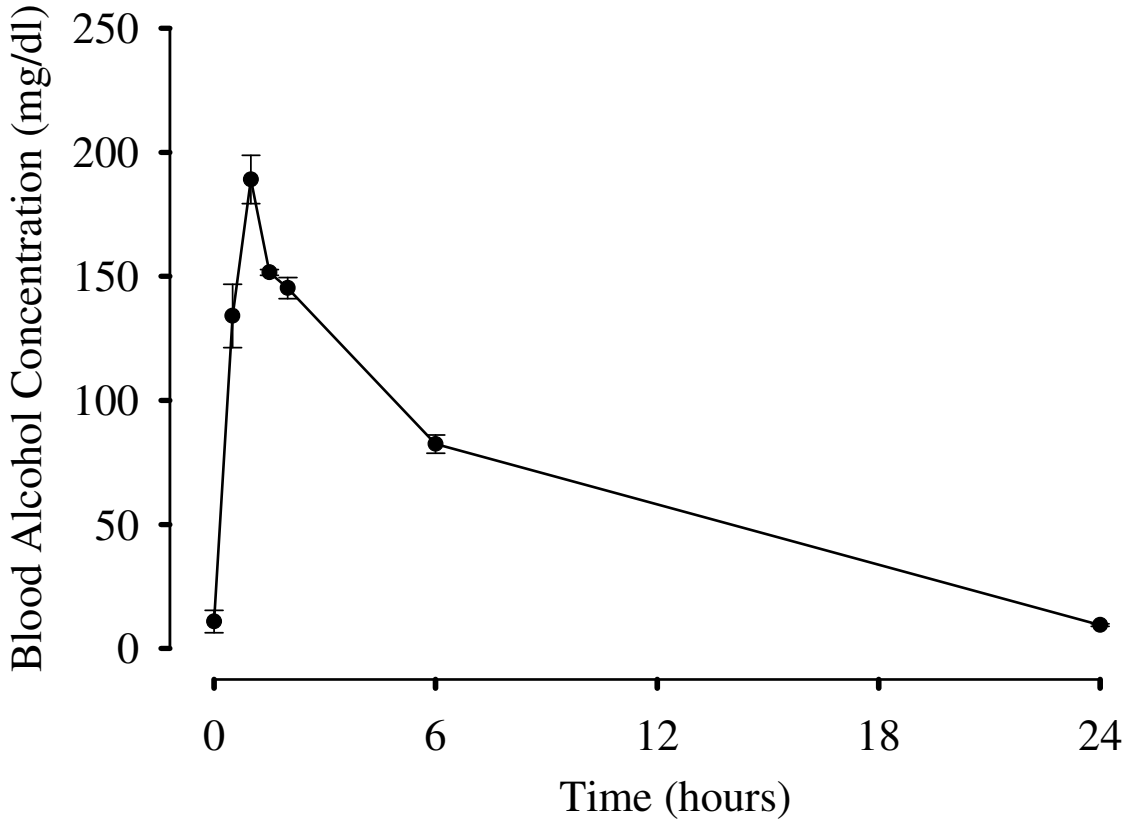

Figure A-6: Maternal blood alcohol concentrations. Maternal blood alcohol concentrations in response to a one hour intravenous infusion of alcohol at a dosage of 1.75g/kg of body weight peaked at 1 hour in the alcohol group. Values measured from samples collected on days 6, 40, 90 and 132 were combined as they were not different from one another. Values are mean $\pm$ SEM.

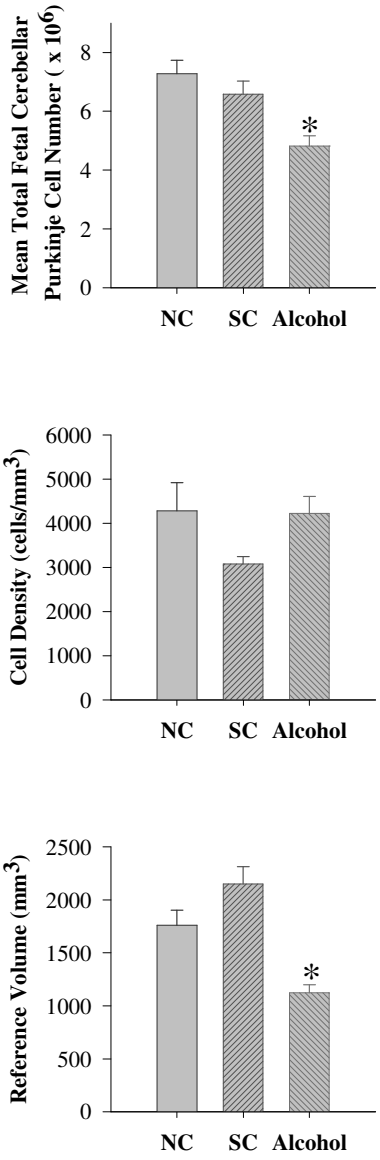

Figure A-7: Effect of all three trimester alcohol on cerebellar Purkinje cells. Estimated total number of fetal cerebellar Purkinje cells (top), cell density (middle) and reference volume (bottom) in normal control (NC), saline control (SC) and alcohol groups. Binging throughout gestation significantly decreased (*) the total number of fetal cerebellar Purkinje cells and the cerebellar reference volume compared with the normal and pair-fed saline control groups. No significant differences were found in the Purkinje cell density. Values are mean $\pm$ SEM.

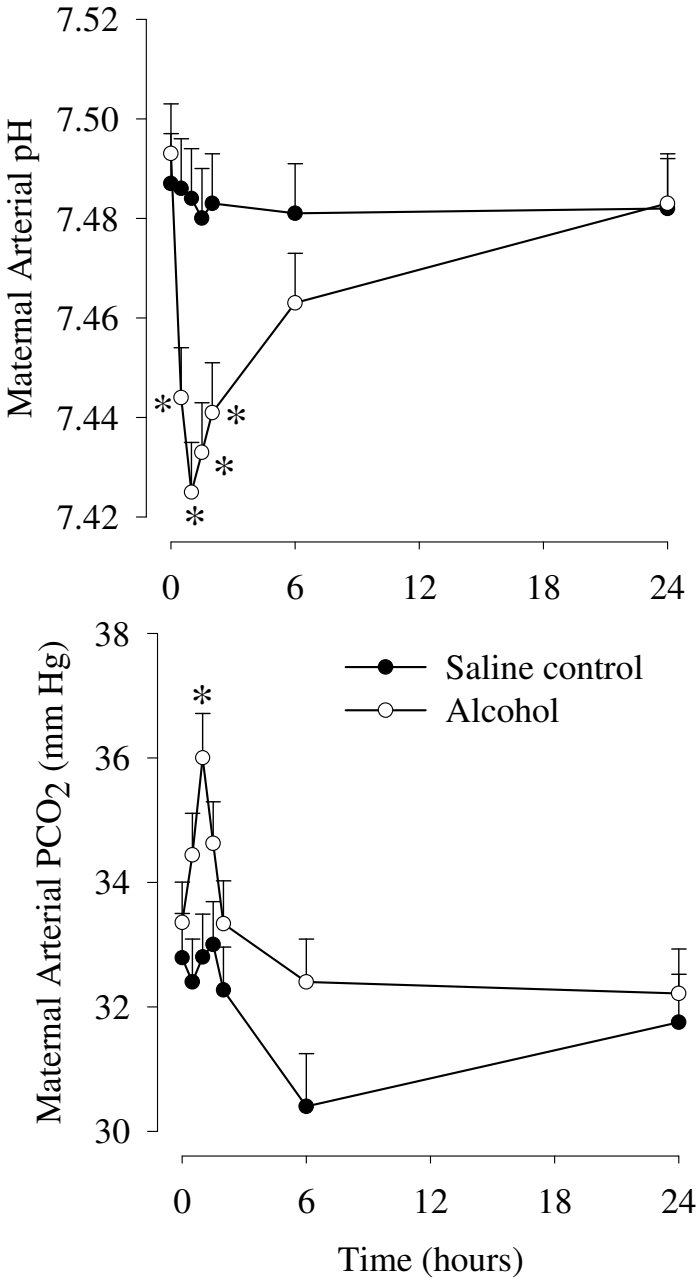

Figure A-8: Maternal arterial pH (top) and P_aCO_2 (bottom) over time. Values measured from samples collected on different days of gestation were combined as they were not different. The pH value at the 1st hour was significantly decreased (*) in the alcohol group ($p < 0.001$) and such decreases persisted at least one hour after the end of infusion. Values are mean $\pm$ SEM.

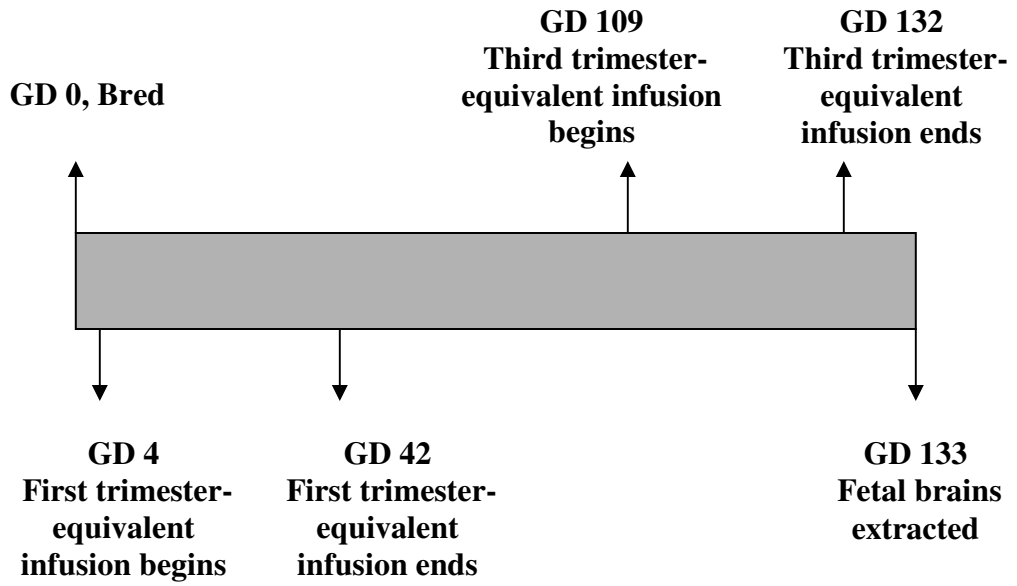

Figure A-9: The experimental paradigm. Alcohol or saline was administered intravenously (IV) over a one-hour period in a binge-like paradigm (modeling weekend only binge drinking), consisting of three consecutive days of exposure followed by four days without exposure and this pattern was repeated throughout the first or third trimester-equivalent (first trimester-equivalent, GD 4-42; third trimester-equivalent, GD 109-132; term is 147 days) and were sacrificed on day 133.

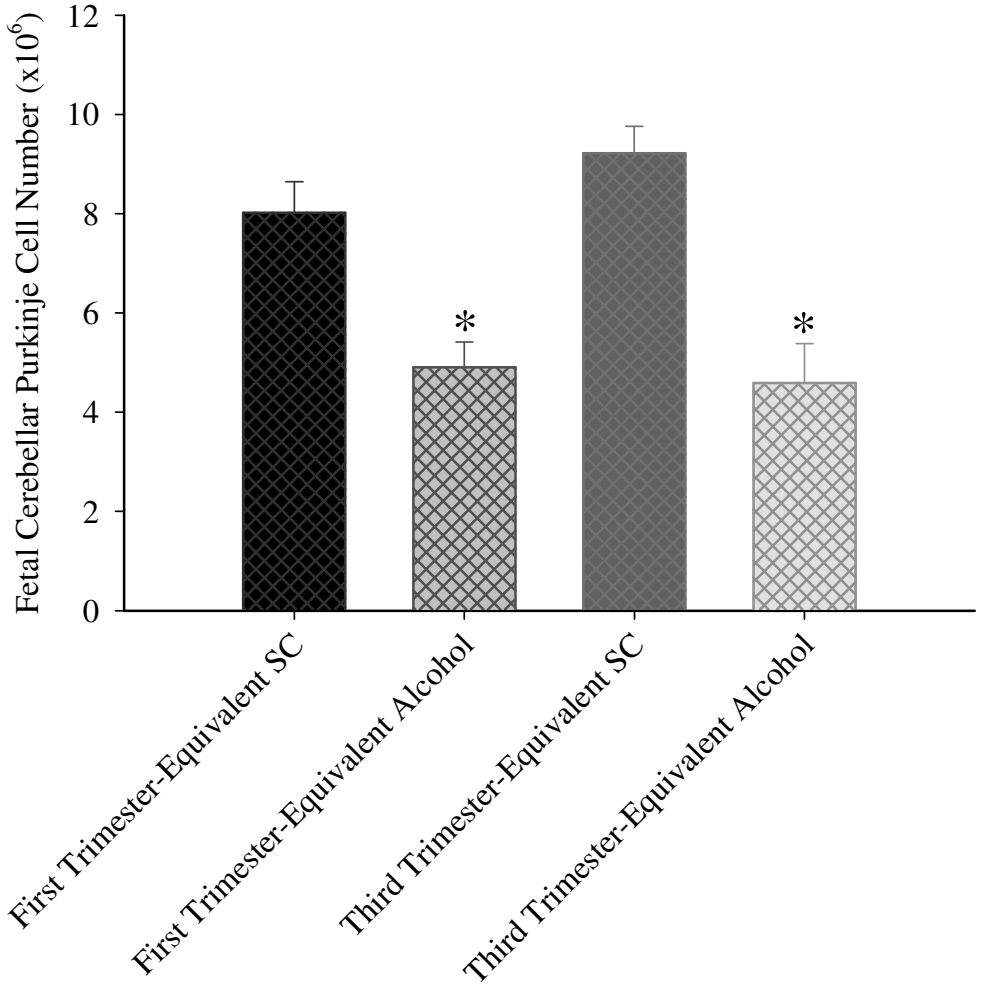

Figure A-10: Estimated total fetal cerebellar Purkinje cell number. Binging during the first or the third trimester-equivalent of gestation significantly decreased (*) the total number of fetal cerebellar Purkinje cells compared with those in the pair-fed saline control subjects. No differences were found between the two alcohol treatment groups. Values are mean + SEM.

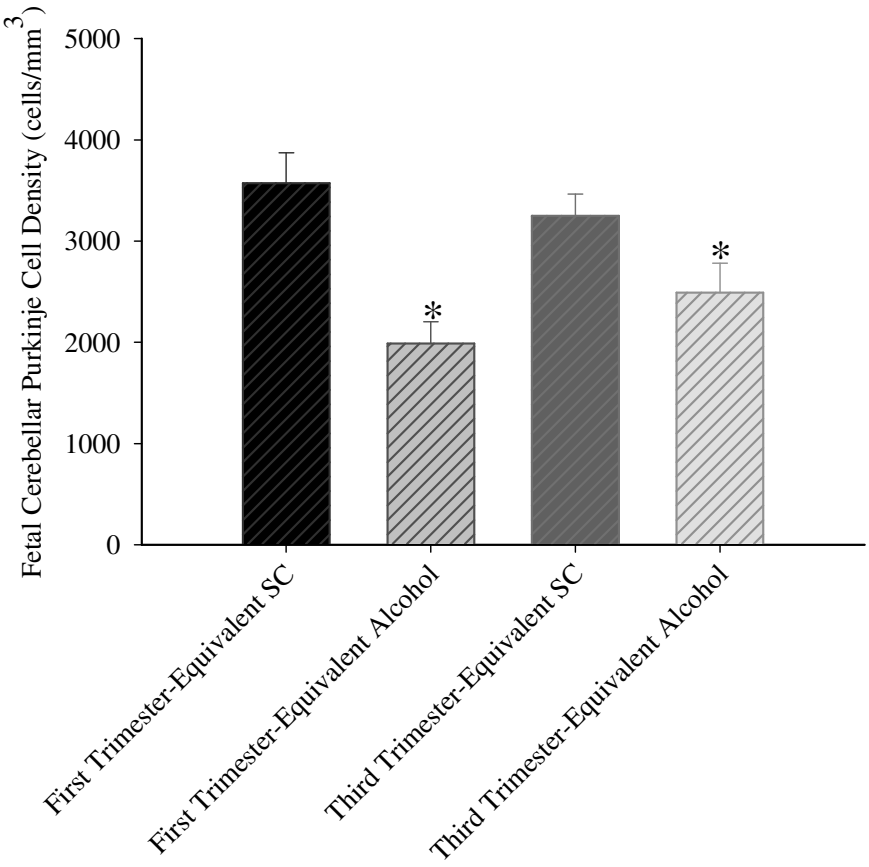

Figure A-11: Fetal cerebellar Purkinje cell density. The fetal cerebellar Purkinje cell density was significantly lower (*) in the alcohol administered groups irrespective of the period during which they received treatment. No differences were found between the first and third trimester-equivalent alcohol treatment groups. Values are mean + SEM.

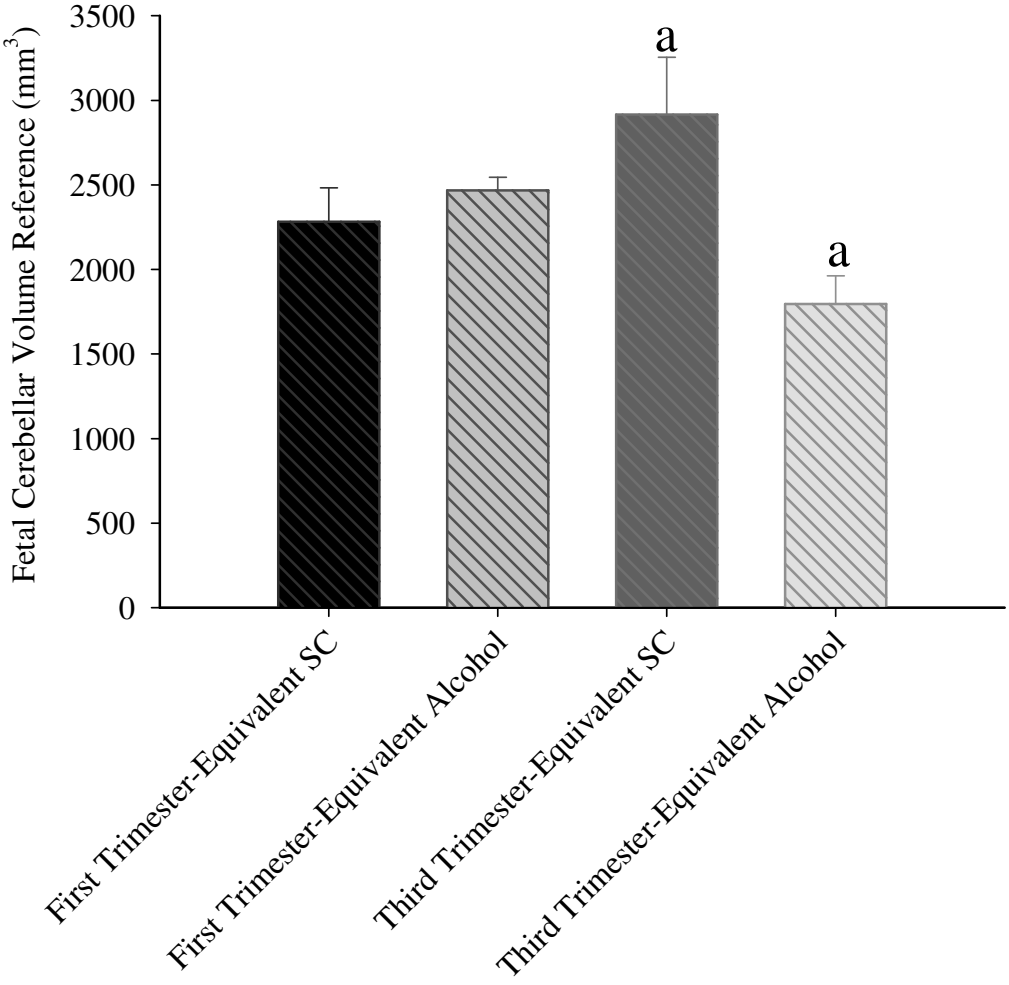

Figure A-12: Fetal cerebellar volume. Third trimester-equivalent alcohol treated subjects had a smaller cerebellar volume reference compared with that in the third trimester-equivalent saline control group (a). Such differences were not found when comparisons were made between the saline controls and the first trimester-equivalent alcohol exposed subjects. Values are mean + SEM.

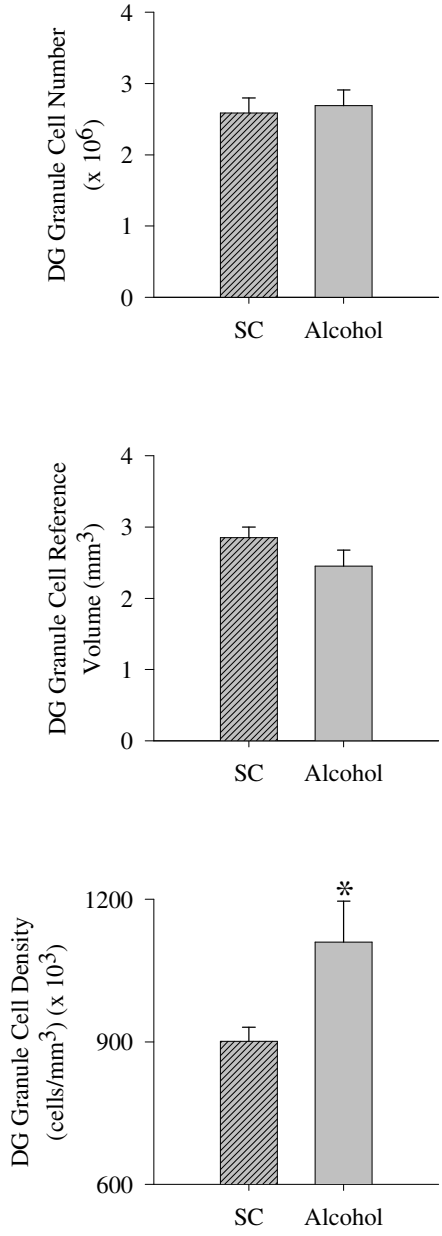

Figure A-13: Dentate gyrus (DG) measurements. The estimated total number of fetal granule cells and the volume of the dentate gyrus were not different between groups. All three trimester binge alcohol exposure significantly increased (*) the packing density of granule cells in the dentate gyrus compared with that in the saline control group (SC). Values are mean $\pm$ SEM.

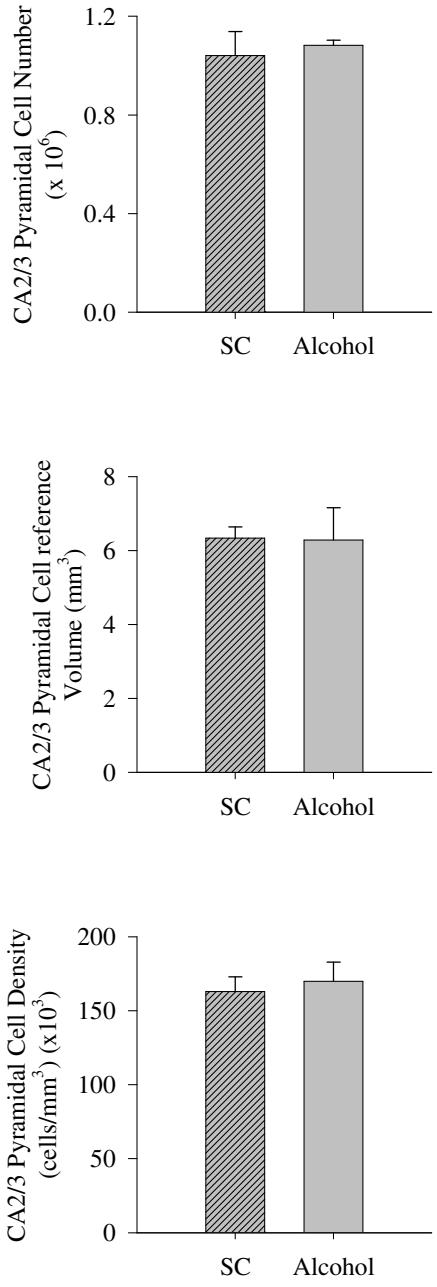

Figure A-14: CA2/3 field measurements. All three trimester-equivalent binge alcohol exposure did not alter fetal hippocampal CA2/3 field measures. The total number of the fetal CA2/3 pyramidal cells, their density, and the volume of the CA2/3 field was not different between the alcohol and the saline control groups (SC). Values are mean $\pm$ SEM.

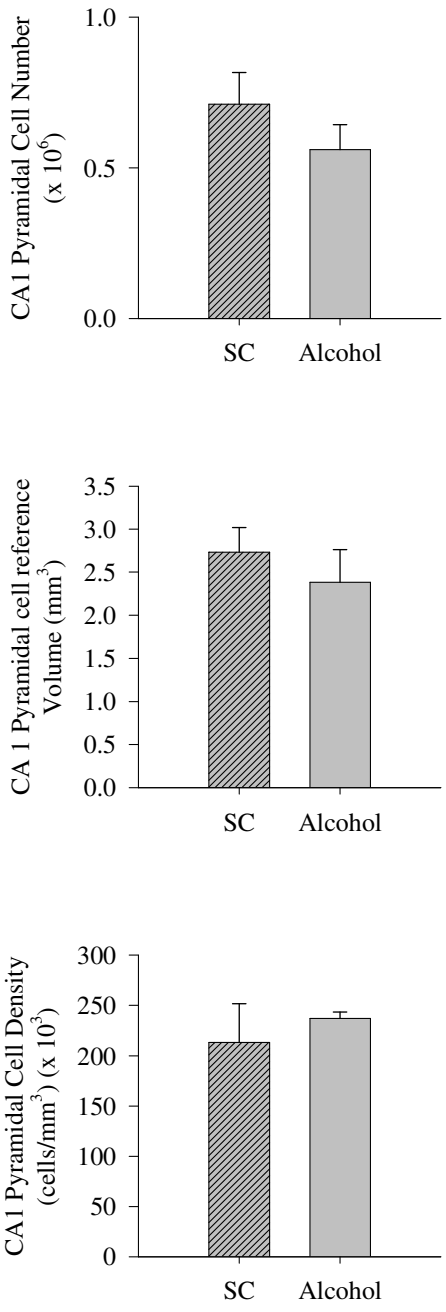

Figure A-15: CA1 field measurements. All three trimester-equivalent binge alcohol exposure did not alter fetal hippocampal CA1 field measures. The total number of the fetal CA1 pyramidal cells, their density, and the volume of the CA1 field was not different between the alcohol and the saline control groups (SC). Values are mean $\pm$ SEM.

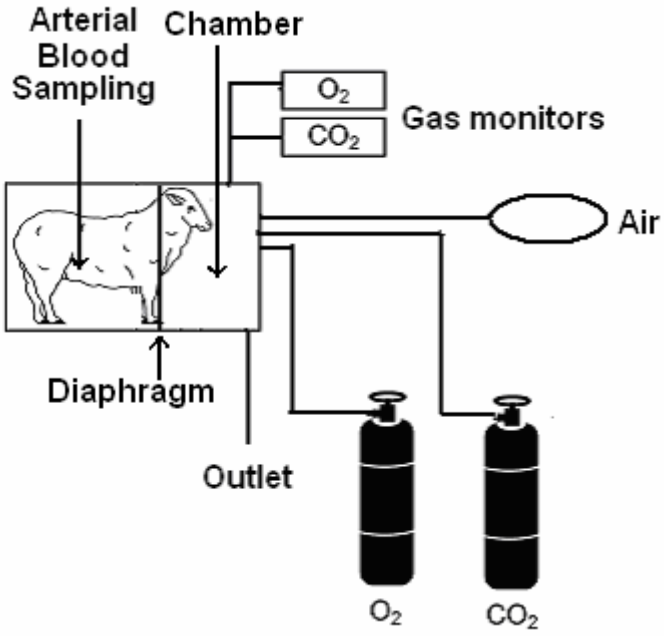

Figure A-16: Illustration of the ventilation chamber. Maternal blood gases in the acidemic group were manipulated for 6 hours by placing the ewe in a chamber and manipulating the inspired gases to mimic the change in maternal arterial pH produced by ethanol. The front half of the subject was confined inside the plexiglass chamber, while the rear half was accessible to the investigator for sampling blood. The fractional concentrations of oxygen and carbon dioxide in the chamber were measured using gas monitors.

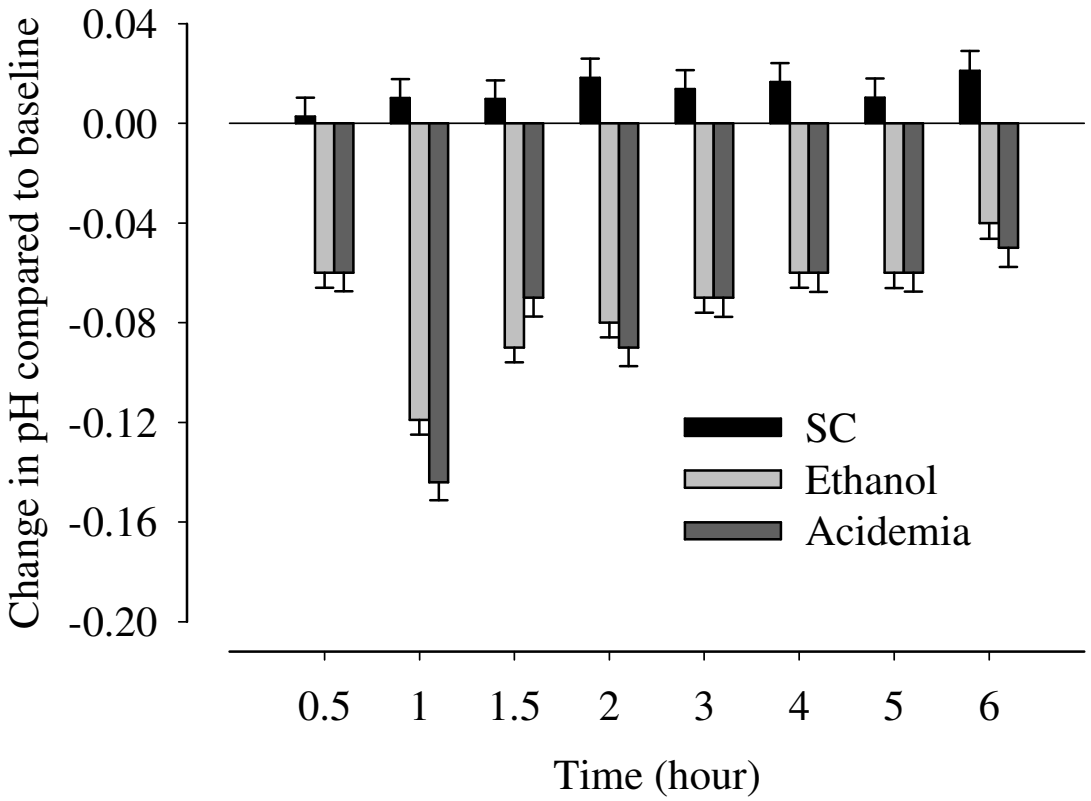

Figure A-17: Maternal arterial pH (pH_a). The magnitude decrease in the maternal pH_a produced by ethanol was created in the acidemic subjects by increasing the inspired partial pressure of carbon dioxide independent of ethanol.

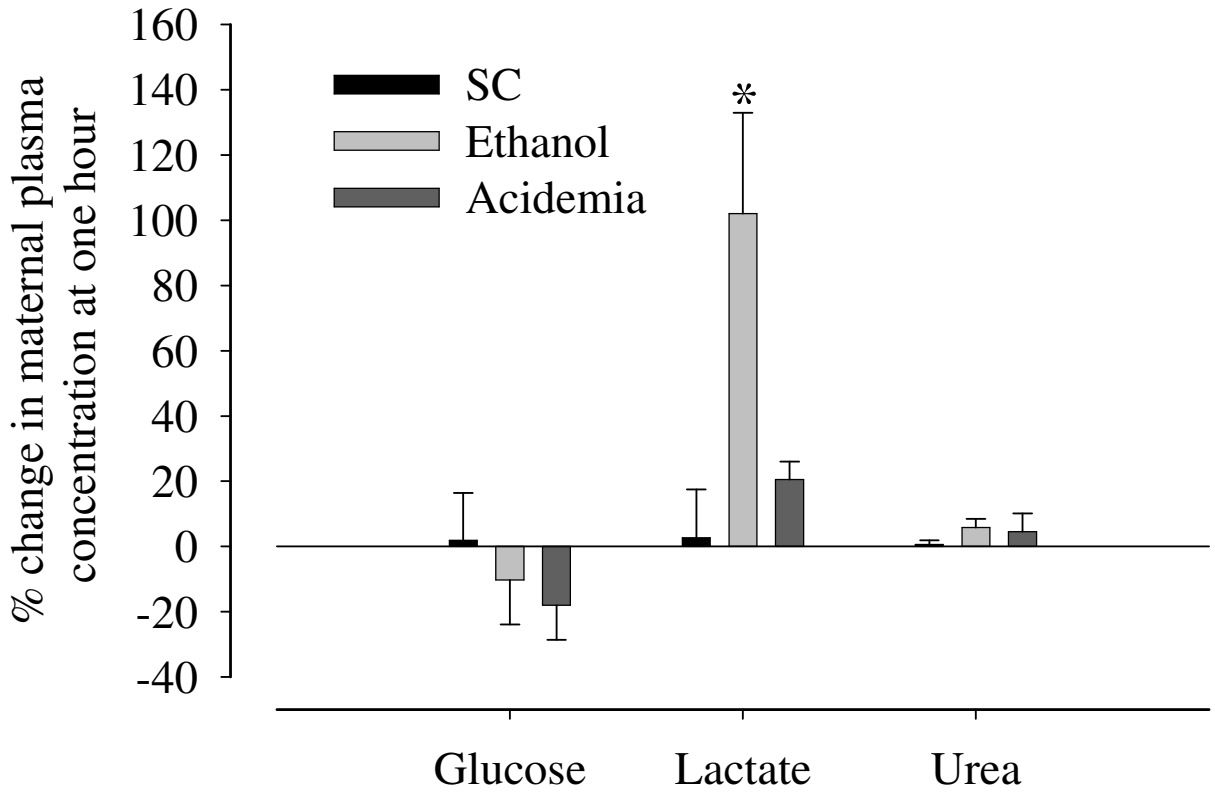

Figure A-18: Maternal plasma glucose, lactate, and urea responses. Maternal plasma glucose, lactate, and urea responsesto 1 hour ethanol, acidemia, or control manipulations following a chronic weekly weekend binge exposure is depicted. Lactate was significantly elevated (*) in the ethanol group compared to the acidemic and saline control (SC) groups. Maternal plasma glucose and urea concentrations were not different when comparing between groups.

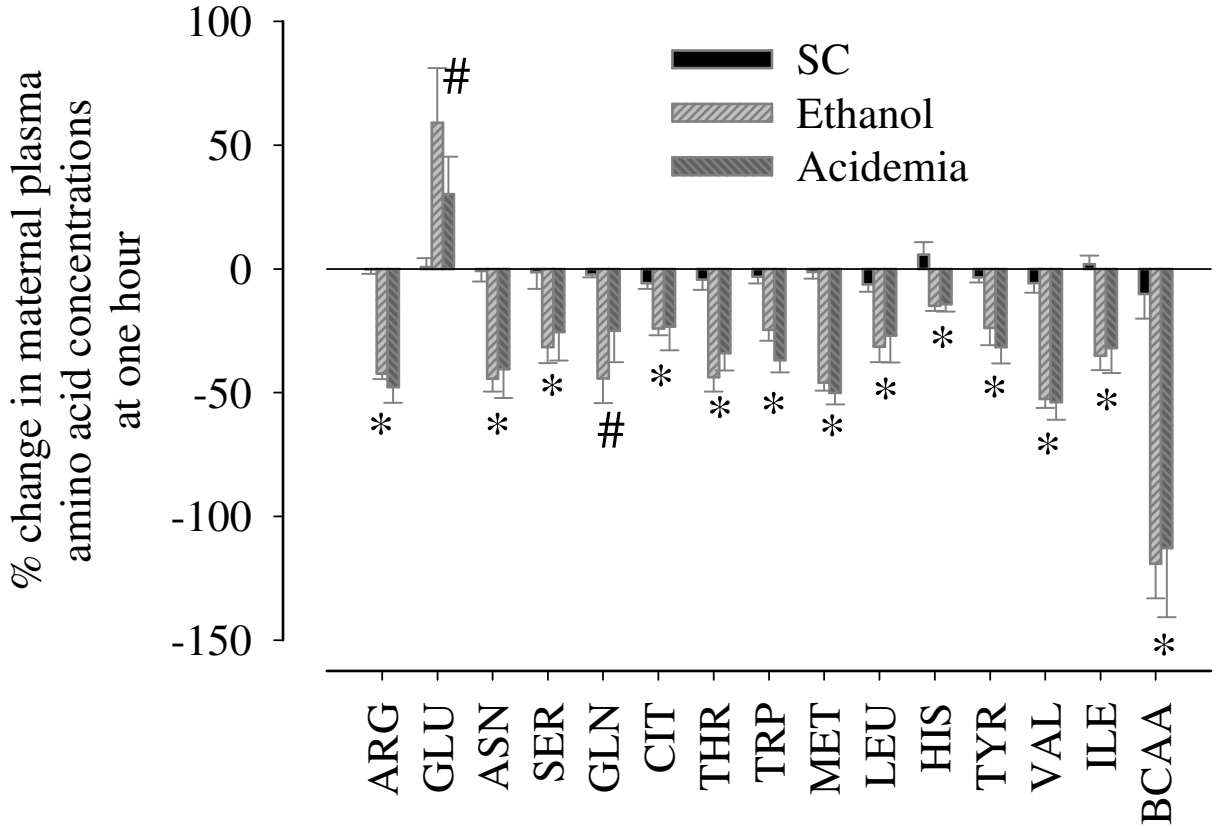

Figure A-19: Maternal plasma amino acid responses. Maternal plasma amino acid responses to 1 hour ethanol, acidemia, or control manipulations following a chronic weekly weekend binge exposure is depicted. Significant reduction (*) in specific amino acids were observed in the ethanol and acidemia groups compared with that in the pair-fed saline control group (SC). Glutamine was significantly decreased (#) in the ethanol group whereas the acidemia group trended lower compared to the control group. The only amino acid that exhibited a significant increase in response to ethanol was glutamate (#). In the acidemia group, glutamate trended higher compared to the control group. No differences were detected between the ethanol and the acidemic groups.

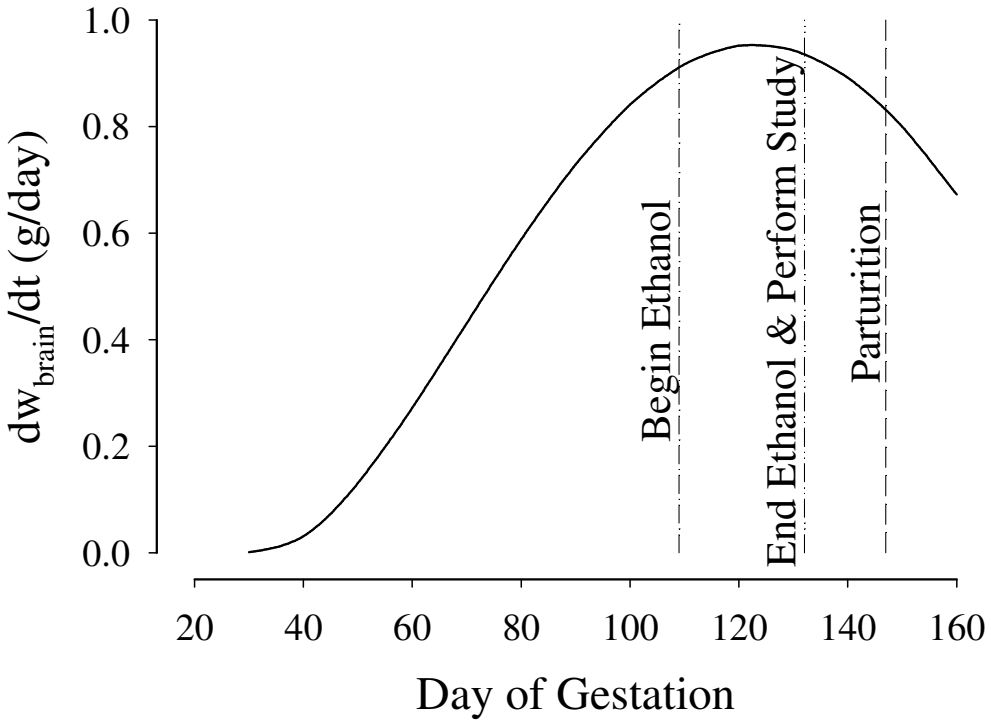

Figure A-20: Fetal sheep brain growth velocity. The ethanol exposure paradigm was designed to occur during the period of peak brain growth velocity like that during the third trimester in humans, a period when it is known that the brain is sensitive to ethanol mediated damage. In order to reasonably extrapolate the timing of exposure from sheep to humans, we differentiated the equation reported by Richardson and Herbert (1978) that predicts ovine fetal brain weight as a function of gestational age, with respect to time, and the first order velocity curve was plotted in a manner similar to that of Dobbing and Sands (1979).

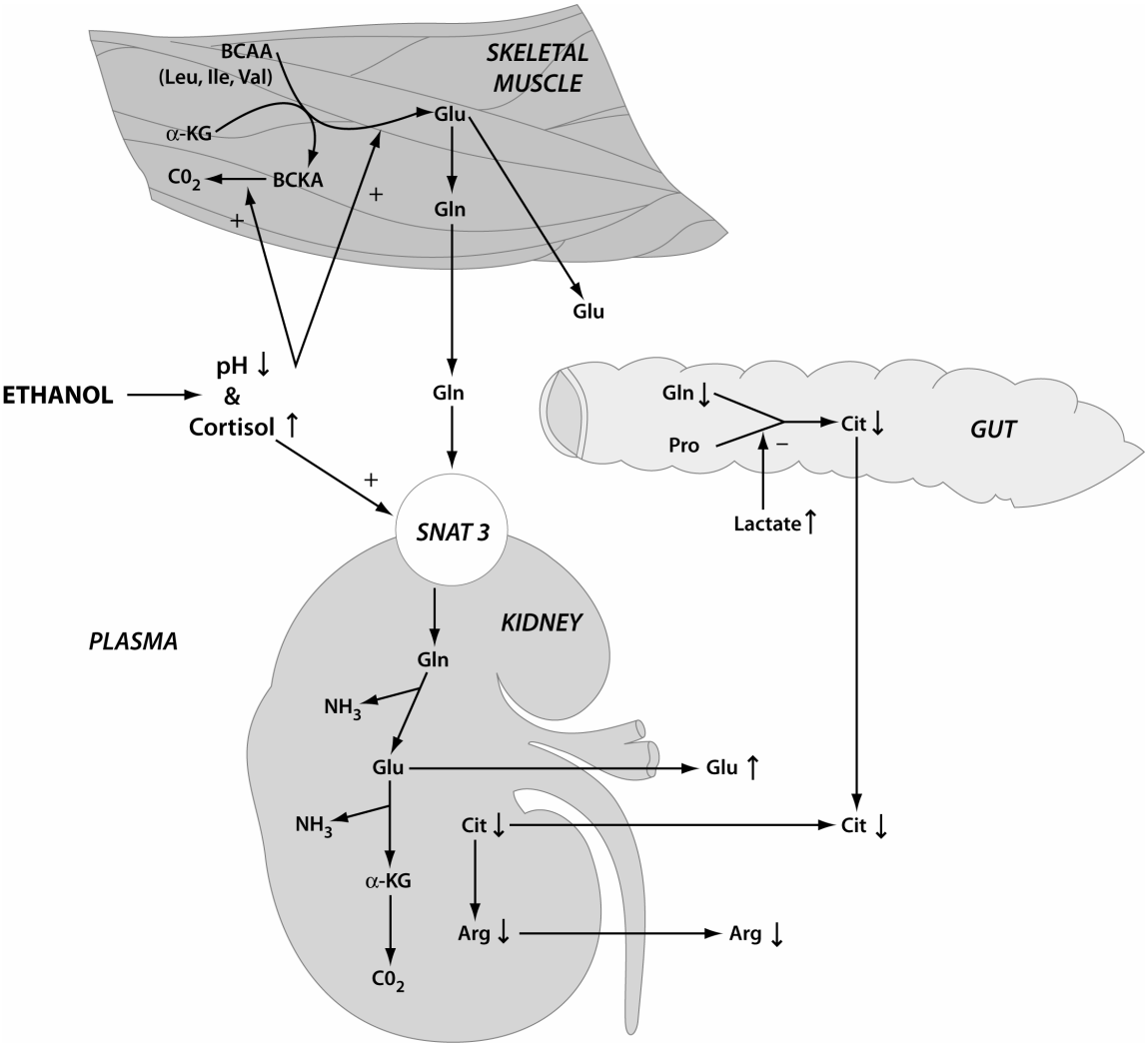

Figure A-21: The ethanol-glutamine model. The ethanol-glutamine model illustrates putative maternal amino acid homeostatic responses to an acute challenge of ethanol following chronic exposure. The kidney acts as a major sink for glutamine during ethanol induced acidosis. Increased renal extraction of plasma glutamine results in decreased availability of arginine and citrulline whose syntheses are glutamine dependent. Acidemia induced elevations in renal glutamine SNAT3 transporter expression result in increased renal uptake of histidine and asparagine. In the muscle, ethanol induced acidosis up-regulates the transamination of branched chain amino acids (BCAA) with α-ketoglutarate to form glutamate and branched chain α-ketoacids (BCKA) and also directly stimulates the oxidative catabolism of BCKAs, leading to decreased plasma levels of BCAAs.

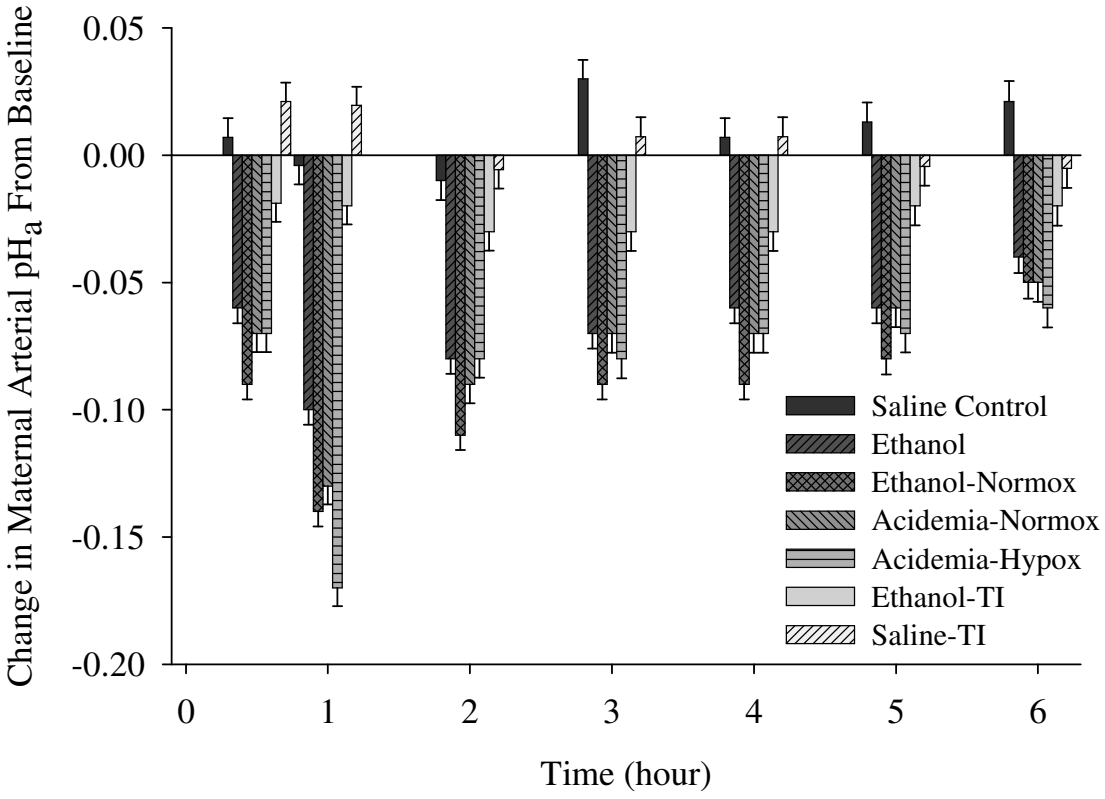

Figure A-22: Maternal acid-base status. The magnitude decrease in the maternal arterial pH (pH_a) produced by ethanol was created in the acidemic-normox and acidemic-hypox subjects, by increasing the inspired partial pressure of carbon dioxide independent of ethanol. In the ethanol-TI group, the TASK inhibitor was infused to prevent ethanol-induced decreases in pH_a.

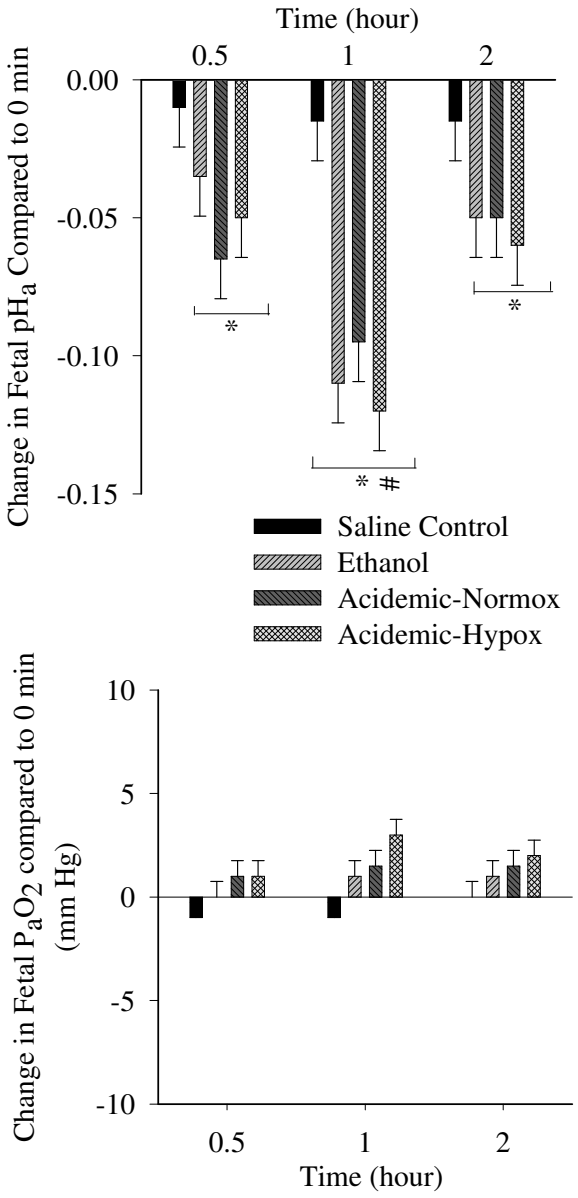

Figure A-23: Fetal blood gases and pH. Fetal arterial pH (pH_a) and partial pressure of oxygen (P_aO_2) are depicted. The fetal pHa was significantly decreased (*) in ethanol, acidemic-normox, and acidemic-hypox groups compared to the pair-fed saline control group. The pHa at the end of the infusion (1 hour) was different (#) from that at all other time points. The fetal P_aO_2 was not different among groups or among time points. Values are mean + SEM.

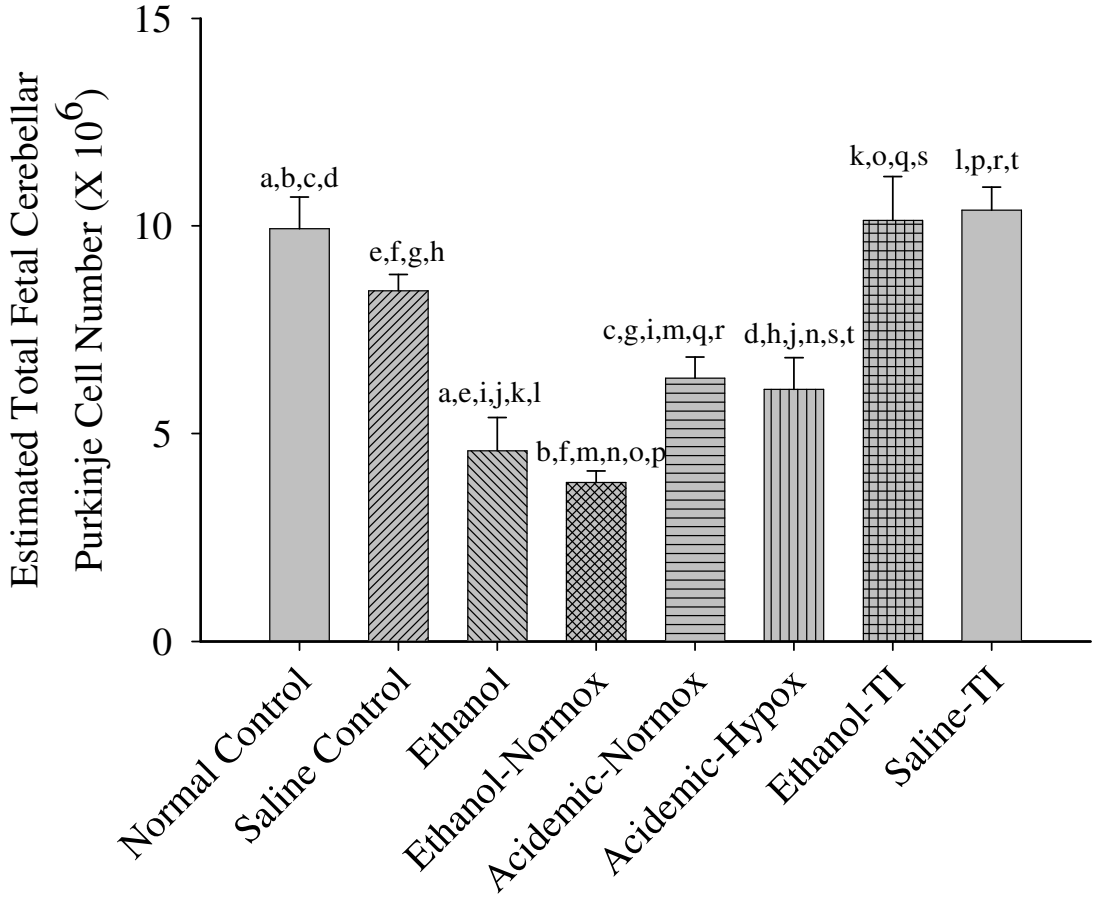

Figure A-24: Effect of ethanol-induced acidosis on Purkinje cell number. The effects of ethanol, acidemia, hypoxemia and TASK inhibition (TI) on the estimated total fetal cerebellar Purkinje cell number. Same letters are different. Values are mean + SEM.

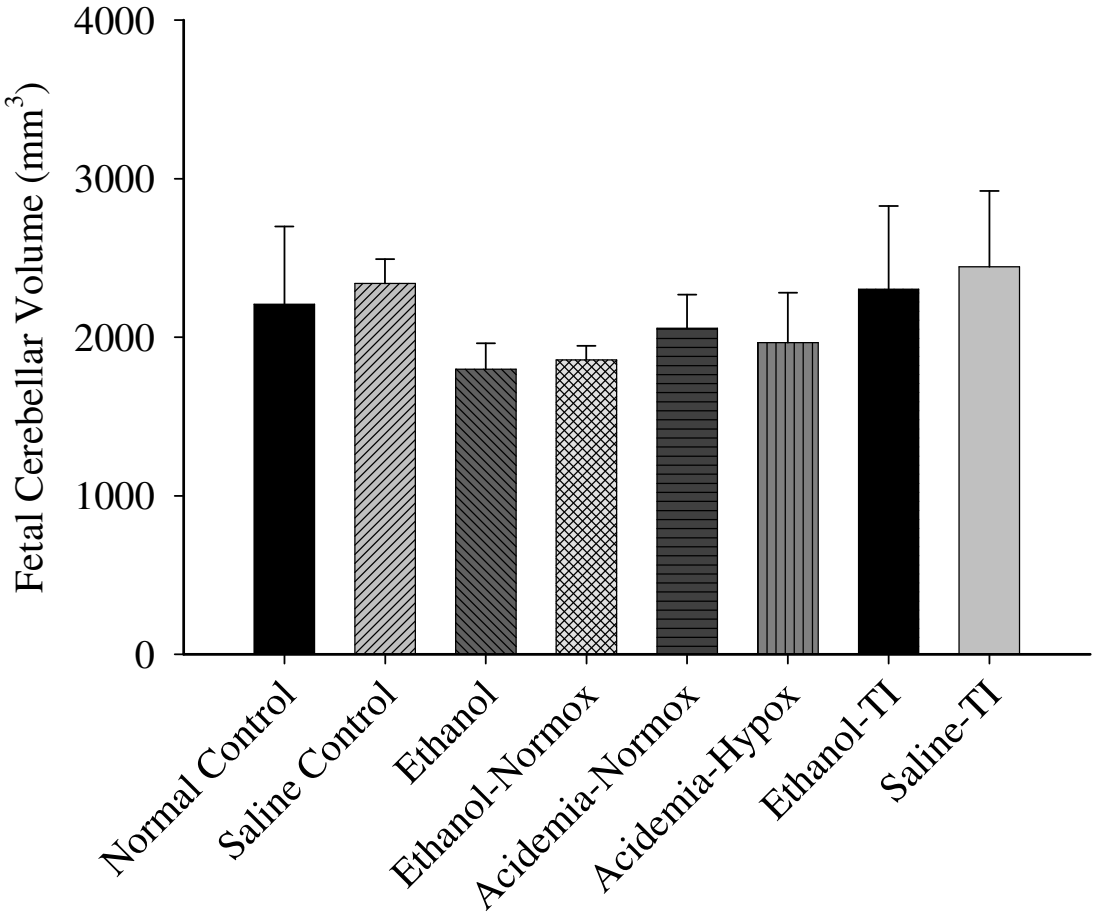

Figure A-25: Effect of ethanol-induced acidosis on cerebellar volume. The effects of ethanol, acidemia, hypoxemia and TASK inhibition (TI) on the cerebellar volume.

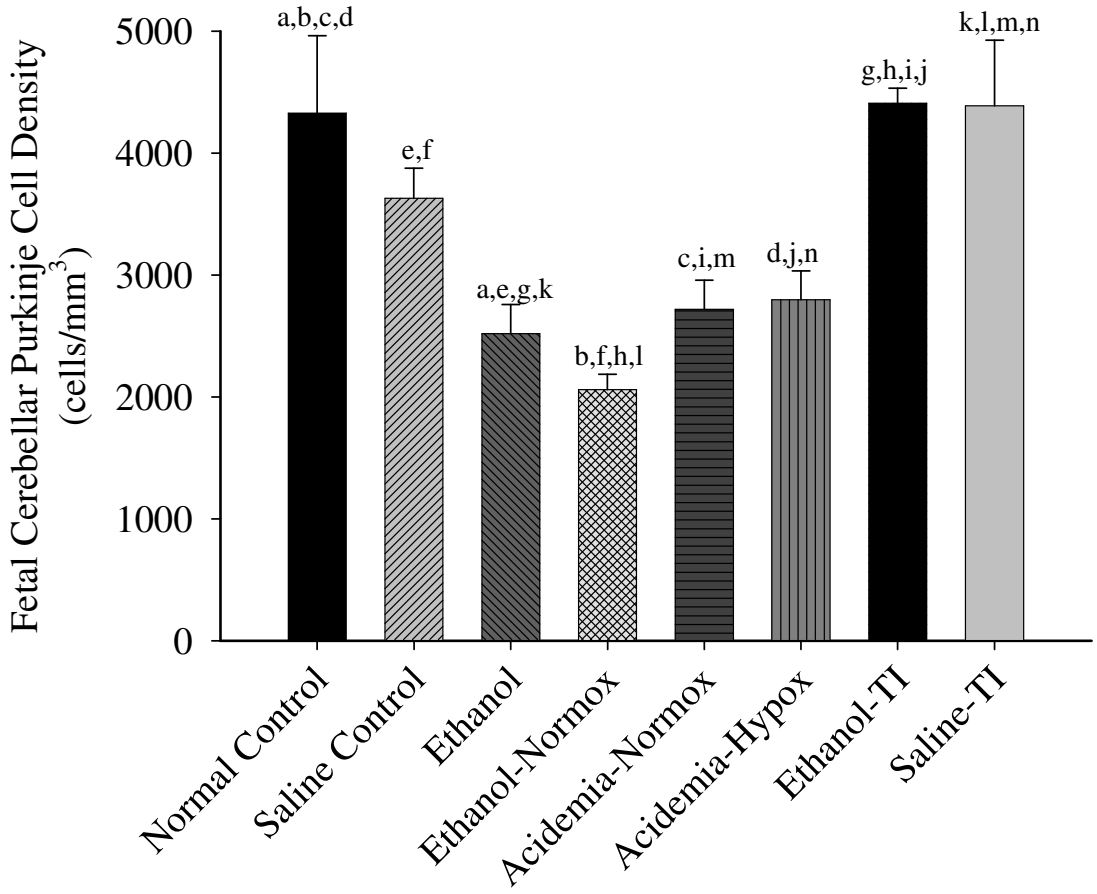

Figure A-26: Effect of ethanol-induced acidosis on cell density. The effects of ethanol, acidemia, hypoxemia and TASK inhibition (TI) on the fetal cerebellar Purkinje cell density. Same letters are different. Values are mean + SEM.

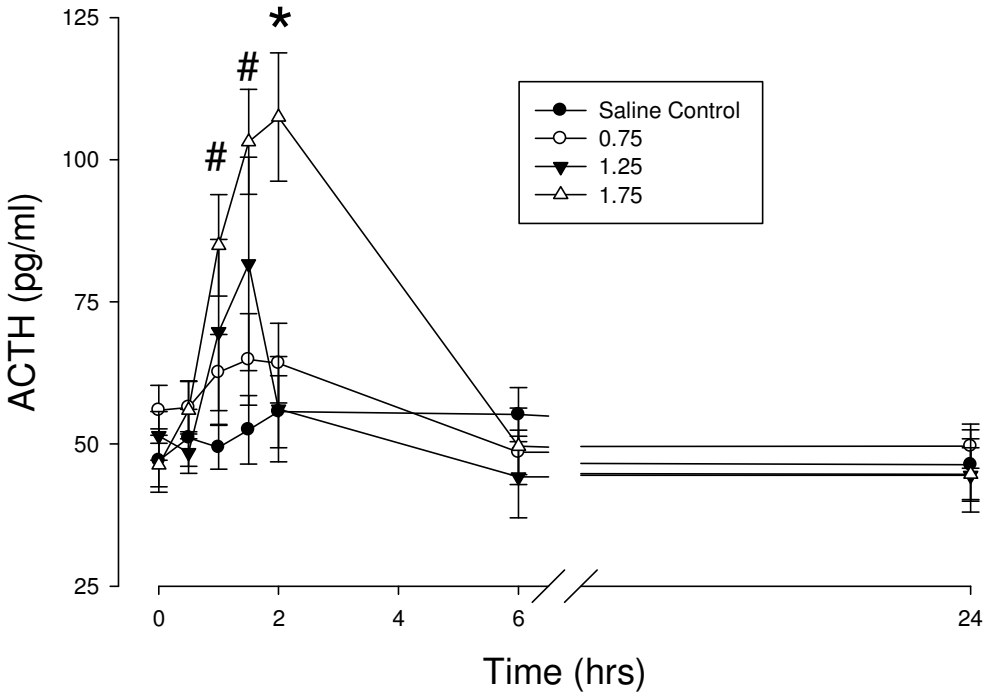

Figure A-27: Maternal plasma ACTH concentrations. Maternal plasma ACTH concentrations (pg/ml ± SEM) were measured from the beginning of the infusion period every 30 min for 2 hours and then at 6 and 24 hours on gestational days (GDs) 6, 40, 90 and 132 and the responses to alcohol were not different when compared between GD. The infusion of 1.75 g/kg of alcohol significantly increased maternal plasma ACTH concentrations at 1 and 1.5 hours compared to all other treatment groups except for the 1.25 g/kg group (#) and compared to all other treatment groups at 2 hours (*).

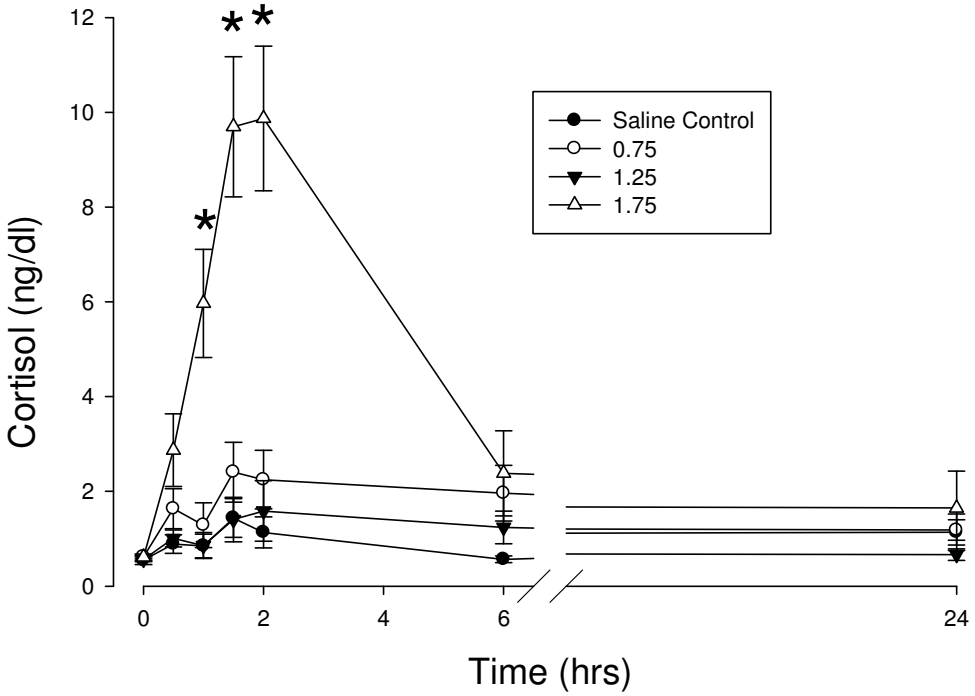

Figure A-28: Maternal plasma cortisol concentrations. Maternal plasma cortisol concentrations (ng/ml ± SEM) were measured from the beginning of the infusion period every 30 min for 2 hours and then at 6 and 24 hours on GDs 6, 40, 90 and 132 and responses to alcohol were not different when compared between GD. The infusion of 1.75 g/kg of alcohol significantly increased maternal plasma cortisol concentrations at 1, 1.5 and 2 hours compared to all other treatment groups (*).

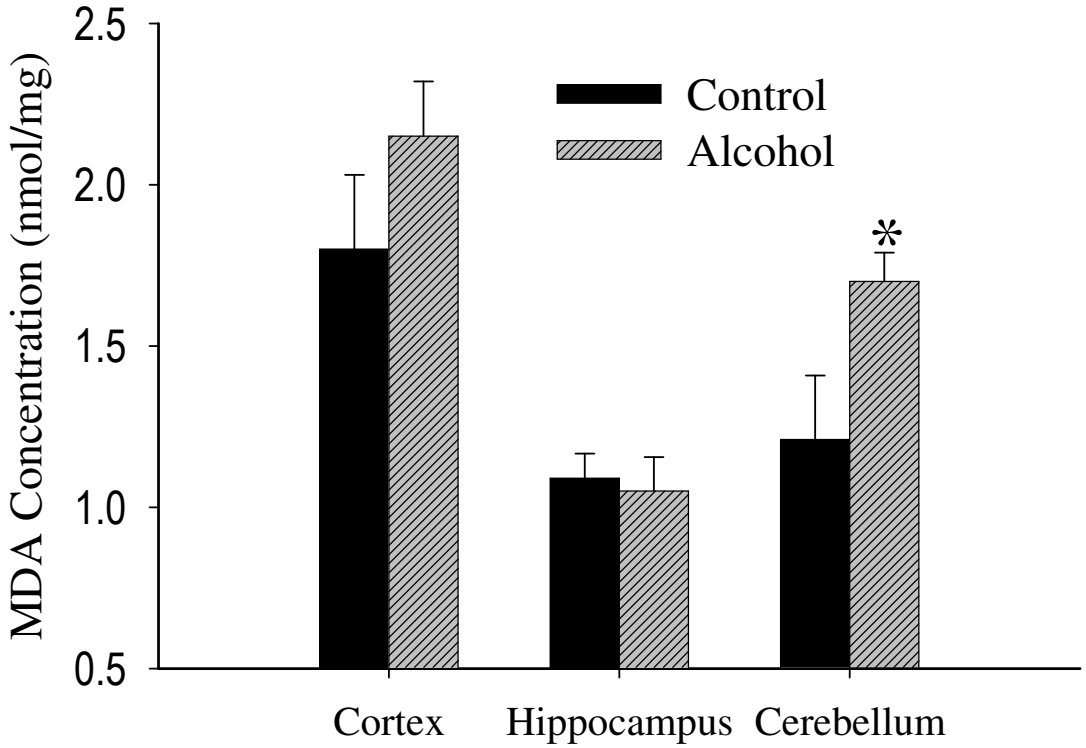

Figure A-29: Regional brain oxidative stress. The concentrations of malondialdehyde (MDA) were examined in the cerebellum, hippocampus, and cortex, and we found that the cerebellum selectively exhibited increased (*) oxidative stress as indicated by the changes in the MDA concentration. A significant change was not observed in the other examined tissues.

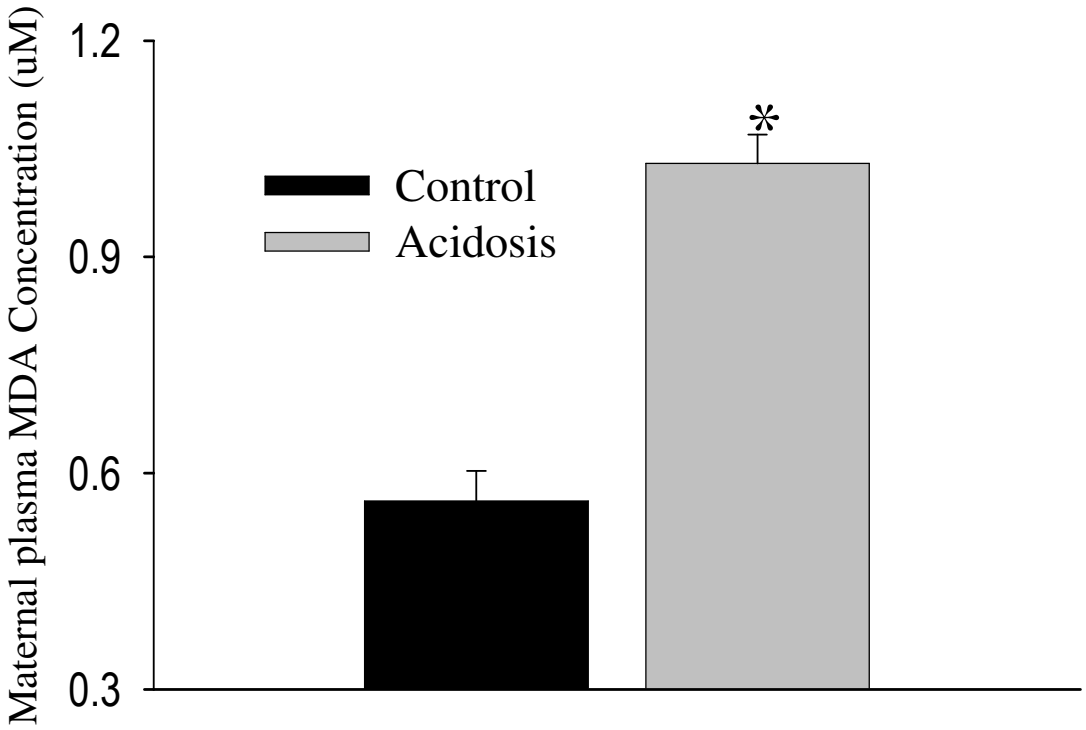

Figure A-30: The effect of acidosis on oxidative stress. The concentration of MDA in the maternal plasma in response to acidosis was examined, and we found that acidosis resulted in significant increases (*) in MDA levels compared to the controls.

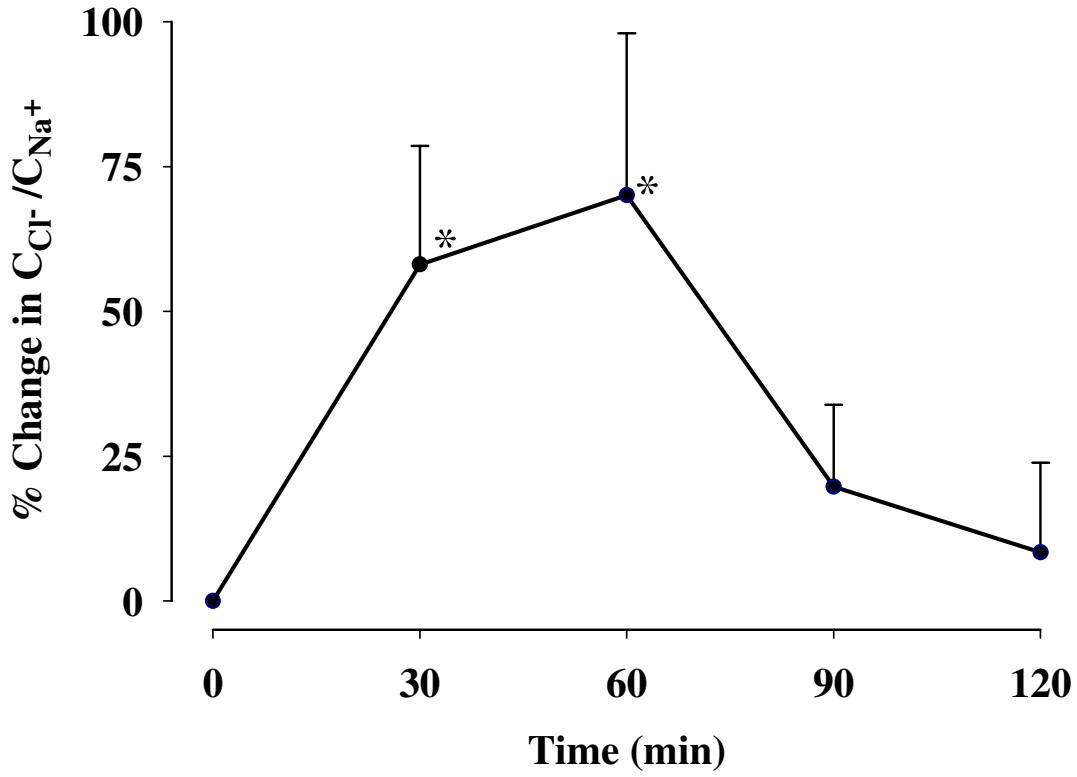

Figure A-31: Acidosis and renal fractional clearance of chloride. Renal fractional clearance of chloride with respect to sodium was significantly increased at 30 and 60 minutes in response to acute respiratory acidosis. The clearance returned to baseline at 90 and 120 minutes when arterial PCO_2 and pH were 37 $\pm$ 0.5 mm Hg and 7.49 $\pm$ 0.1 respectively.

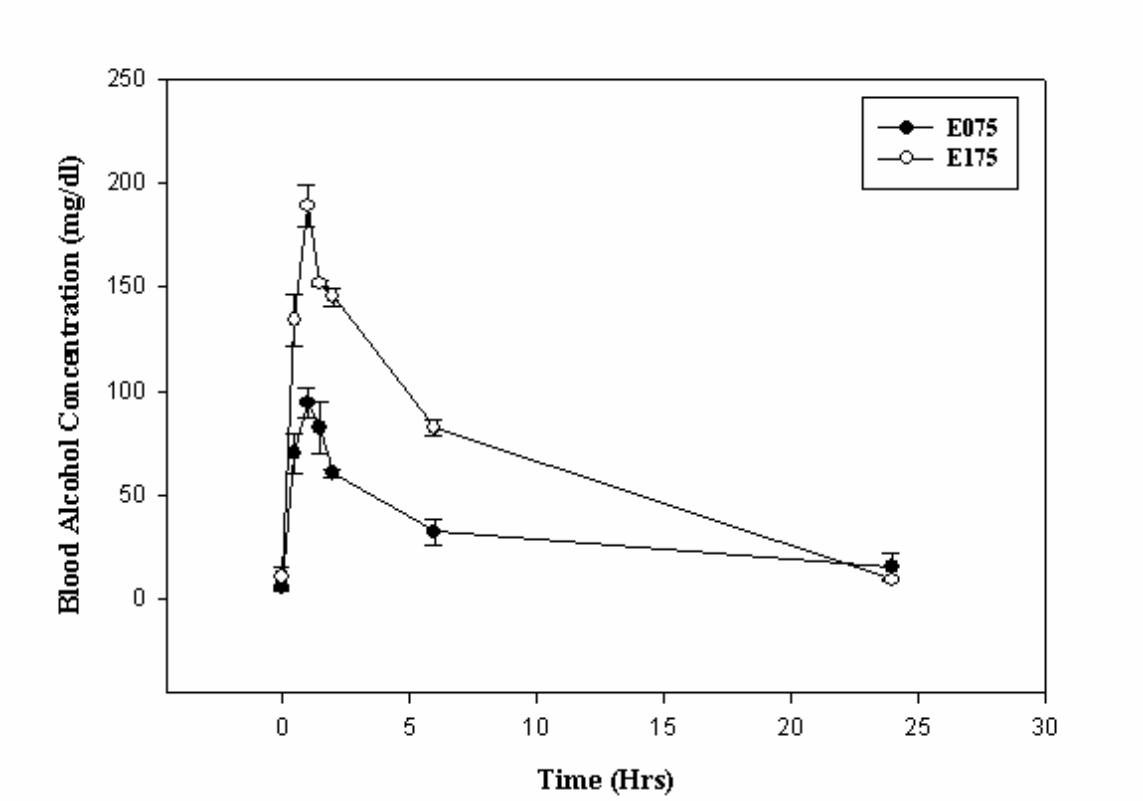

Figure A-32: Maternal blood alcohol concentrations. Maternal blood alcohol concentrations in response to one hour intravenous infusion of alcohol. Mean blood alcohol concentrations peaked at 1 hour for both the lower dose (E075) and higher dose (E175) groups. Values measured from samples collected on days 6, 40, 90 and 132 were combined as they were not different from one another. E075, E175: alcohol dosage 0.75 and 1.75 g/kg of body weight respectively. Values are mean $\pm$ SEM.

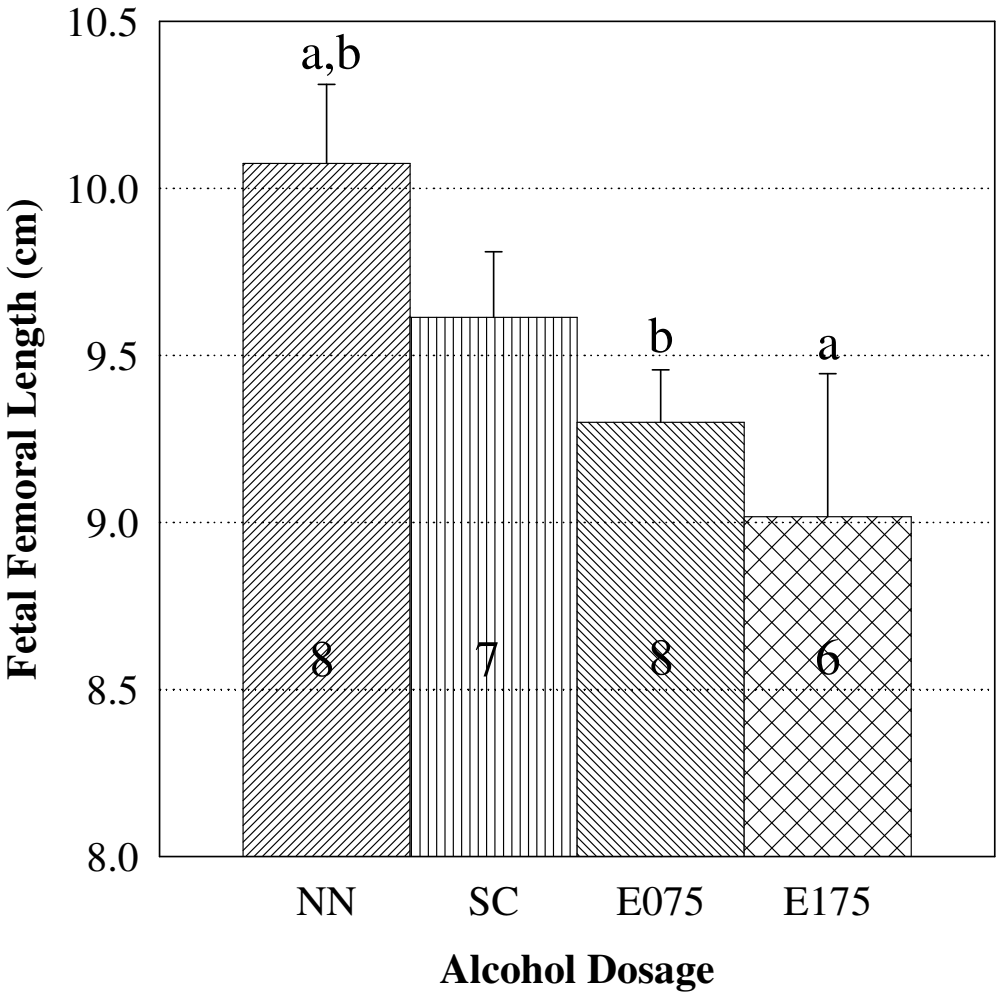

Figure A-33: Effect of alcohol binging on fetal femoral length. Effect of alcohol binging throughout gestation on fetal femoral length is depicted. Fetal femurs in the higher dose group (E175) were shorter than the normal control group (a: $p = 0.039$) with the lower dose group (E075) showing a trend toward shorter lengths (b: $p = 0.079$). The alcohol groups were not different from the saline control group. NN: normal control group; SC: saline control group; E075, E175: alcohol dosage 0.75 and 1.75 g/kg of body weight respectively. Values are mean $\pm$ SEM. Values given in the bars represent the number of bones. Groups sharing the same letter are significantly different in bone length.

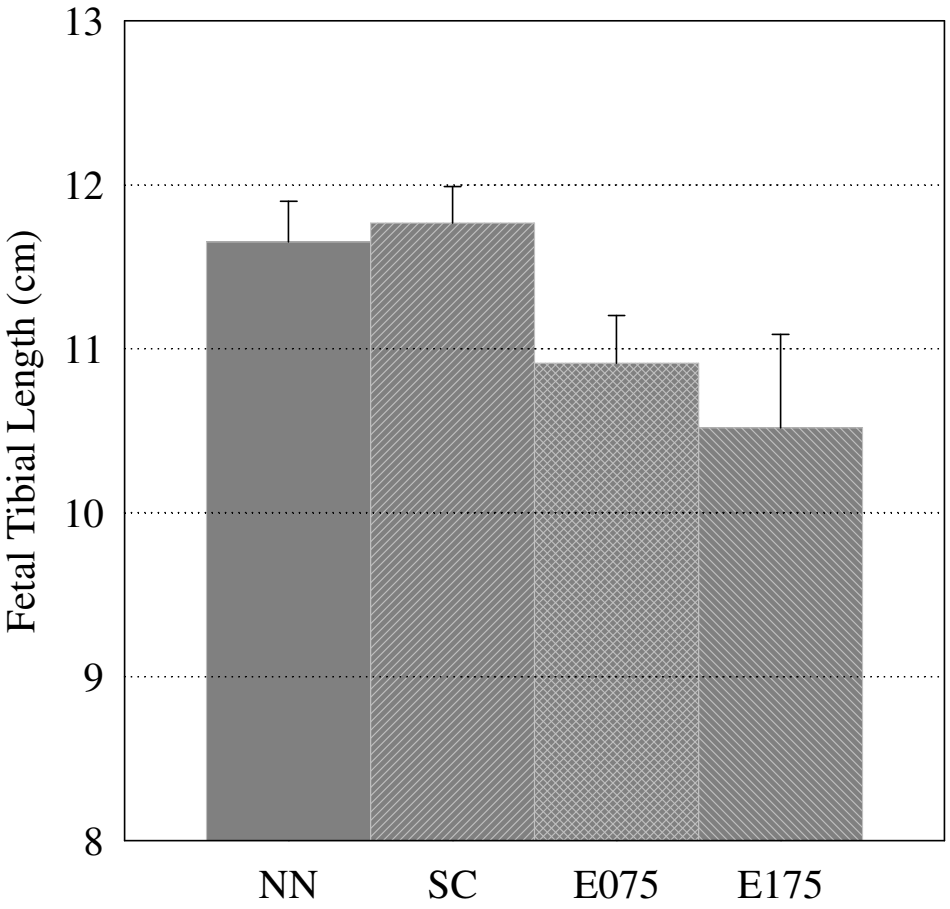

Figure A-34: Effect of alcohol consumption on fetal tibial length. Effect of alcohol consumption throughout gestation on fetal tibial length is depicted. Mean fetal tibial lengths did not exhibit dose-dependent changes (one-way ANOVA, $p = 0.057$). However, the alcohol groups, when combined, showed significantly shorter mean lengths compared to the normal control group (one-way ANOVA, $p = 0.030$). NN: normal control group; SC: saline control group; Alcohol: alcohol dosage groups (0.75 and 1.75 g/kg of body weight) when combined. Values are mean $\pm$ SEM. Values given in the bars represent the number of bones.

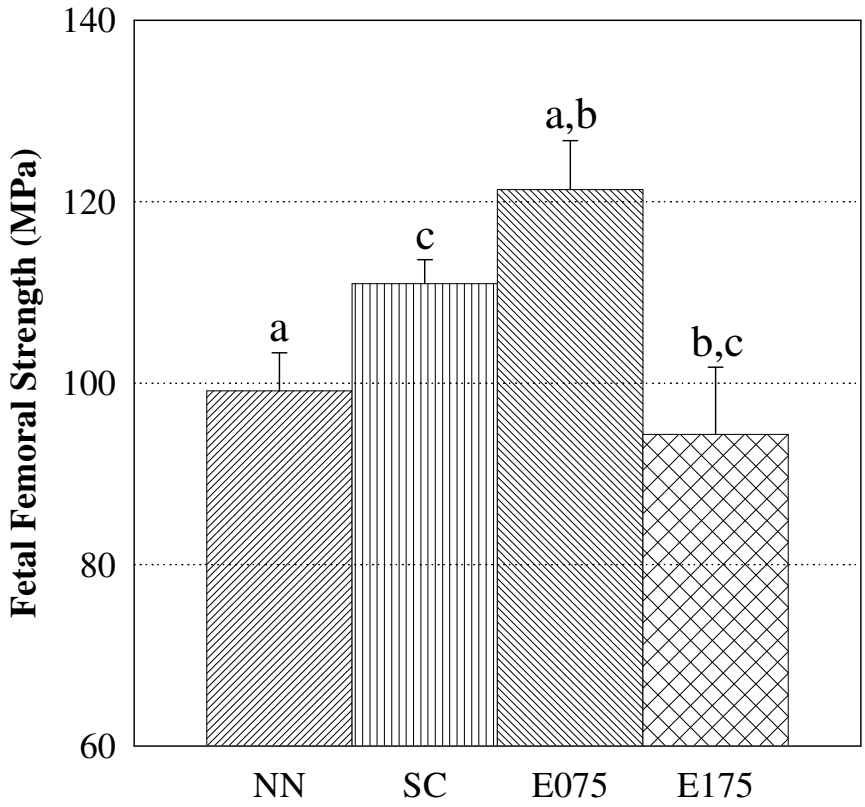

Figure A-35: Effect of moderate and heavy binging on fetal femoral strength. The lower dose group (E075) showed significantly higher bone strength compared to the normal control group (a: p = 0.008). The higher dose (E175) group had lower femoral strength compared to the lower dose (E075) group (b: p = 0.006) and trended lower compared to the saline control group (c: p = 0.089). NN: normal control group; SC: saline control group; E075, E175: alcohol dosage 0.75 and 1.75 g/kg of body weight respectively. Values are mean $\pm$ SEM. Values given in the bars represent the number of bones. Groups sharing the same letter are significantly different in bone strength.

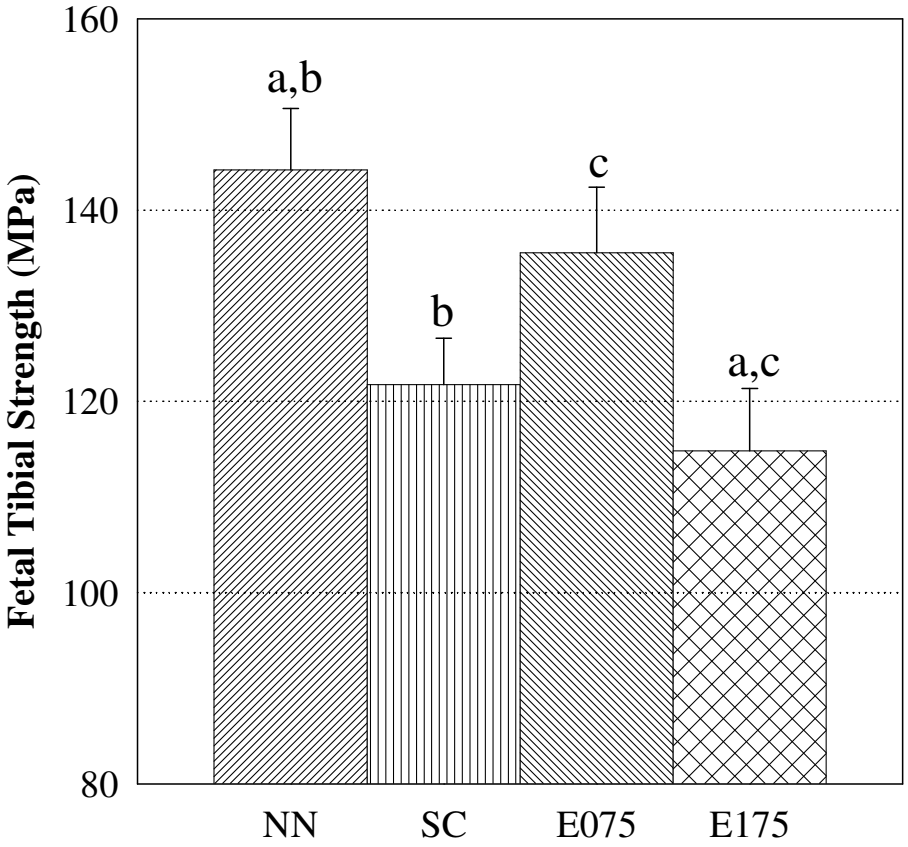

Figure A-36: Effect of moderate and heavy binging on fetal tibial strength. The higher dose group exhibited significantly lower strength compared to the normal control group (a: $p = 0.019$) and trended lower than the lower dose group (c: $p = 0.082$). The saline control group trended to have a lower strength compared to the normal control group (b: $p = 0.056$).NN: normal control group; SC: saline control group; E075, E175: alcohol dosage 0.75 and 1.75 g/kg of body weight respectively. Values are mean $\pm$ SEM. Values given in the bars represent the number of bones. Groups sharing the same letter are significantly different in bone strength.

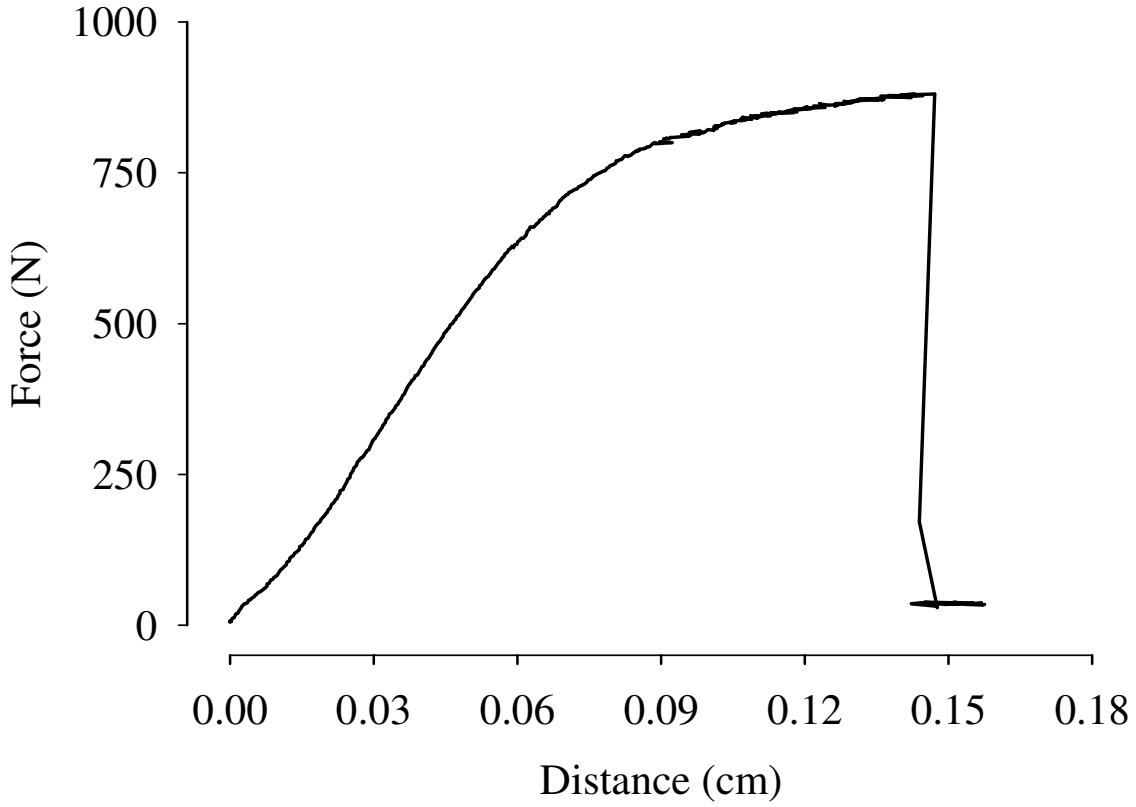

Figure A-37: Representative illustration of a tibial test. Representative illustration of a tibial test using a three point bending procedure is depicted. Force was applied through a single upper contact that was centered between the two lower supports, the maximum force and the displacement were recorded for each bone during the test. Data were analyzed to determine both extrinsic (whole bone), and intrinsic (of the bone tissue) properties.

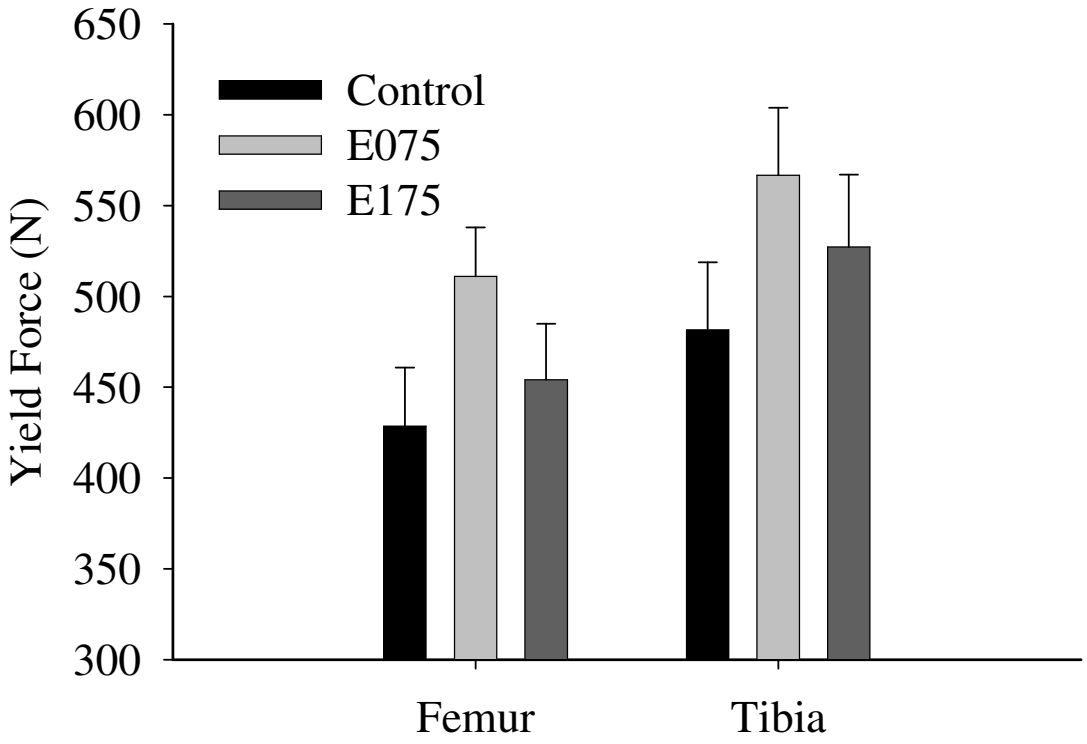

Figure A-38: The yield force. The yield force (N) was defined to be the point of intersection between the force curve and a straight line with a slope of 90% of the stiffness. First trimester maternal alcohol binging did not effect the yield force recorded during the mechanical test for both the fetal femur and tibia.

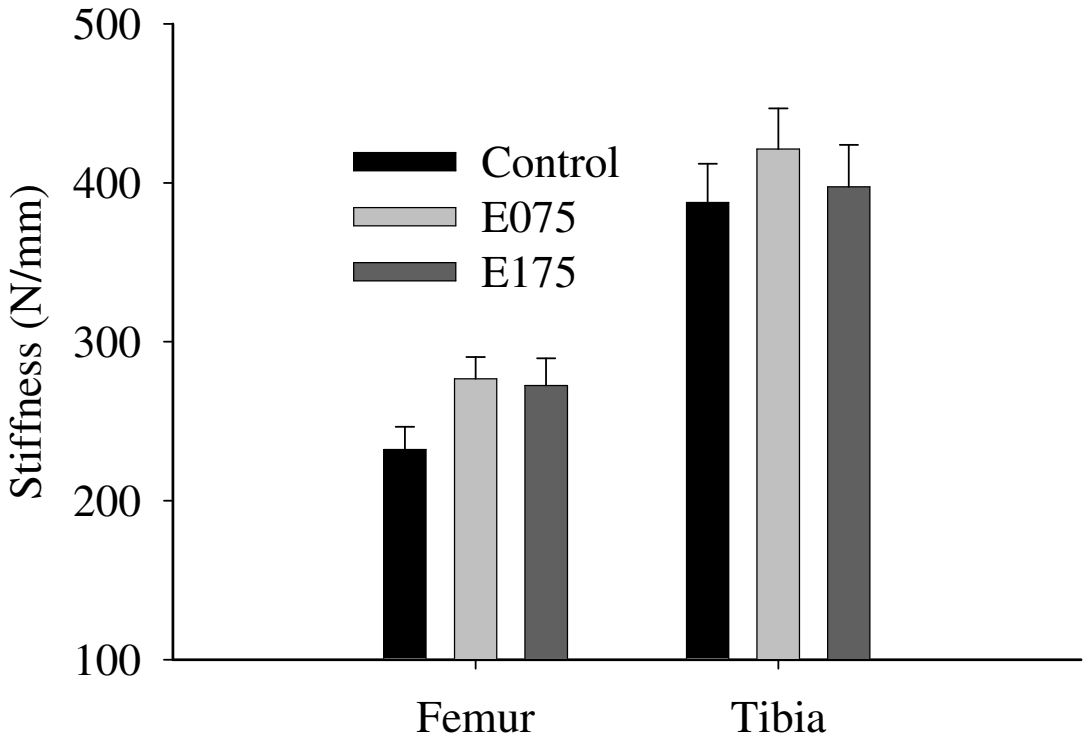

Figure A-39: Bone stiffness. First trimester fetal alcohol exposure did not alter the fetal femoral and tibial stiffness (N/mm). Stiffness was calculated from the slope of the force-displacement curve in the linear region and was determined from linear regression analysis.

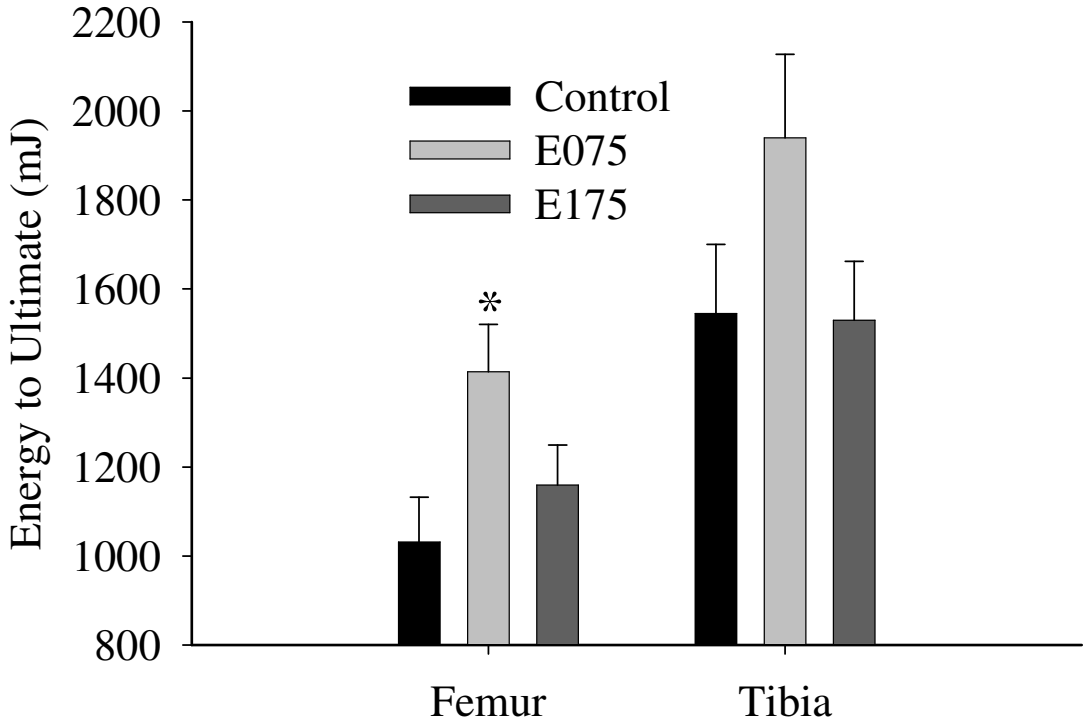

Figure A-40: The energy absorbed by the fetal bones. The total energy (mJ) absorbed by the fetal femur to the ultimate point was significantly higher (*) for the E075 (0.75 g/kg) group compared with that in the control group. No differences were observed for the fetal tibia. This value was calculated as the area under the force displacement curve.

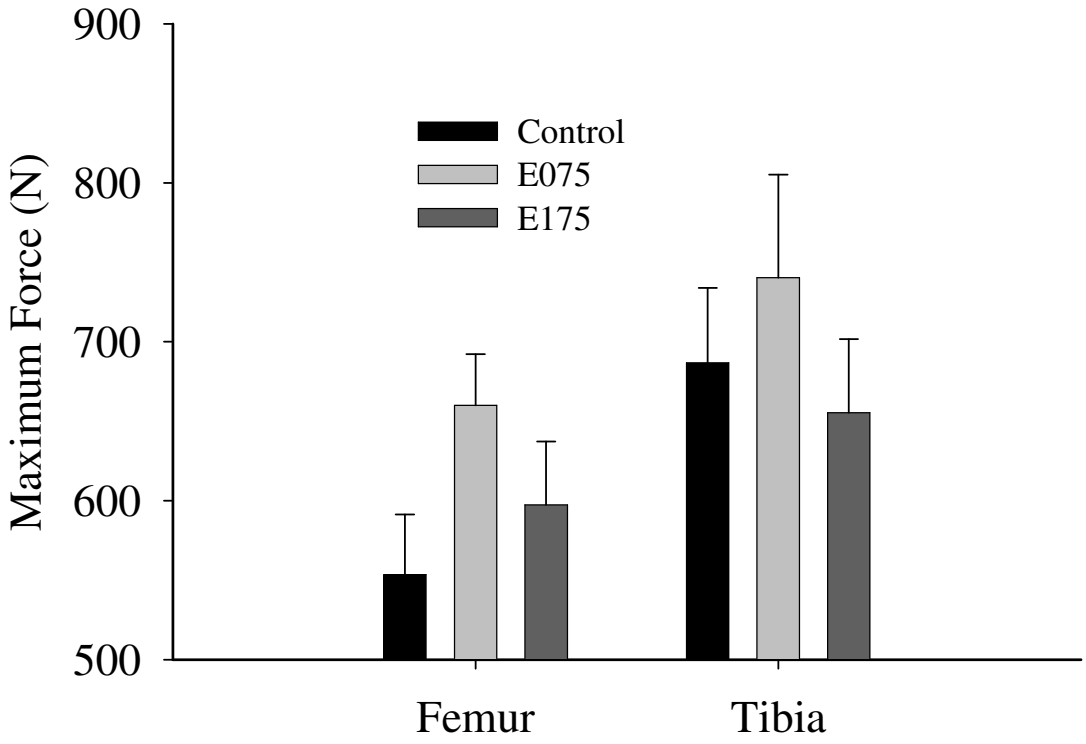

Figure A-41: Maximum force. For all of the fetal bones, there were no significant differences among groups for the maximum force (N). This value is an extrinsic property and is indicative of the contributions of both tissue mineralization and bone size and shape.

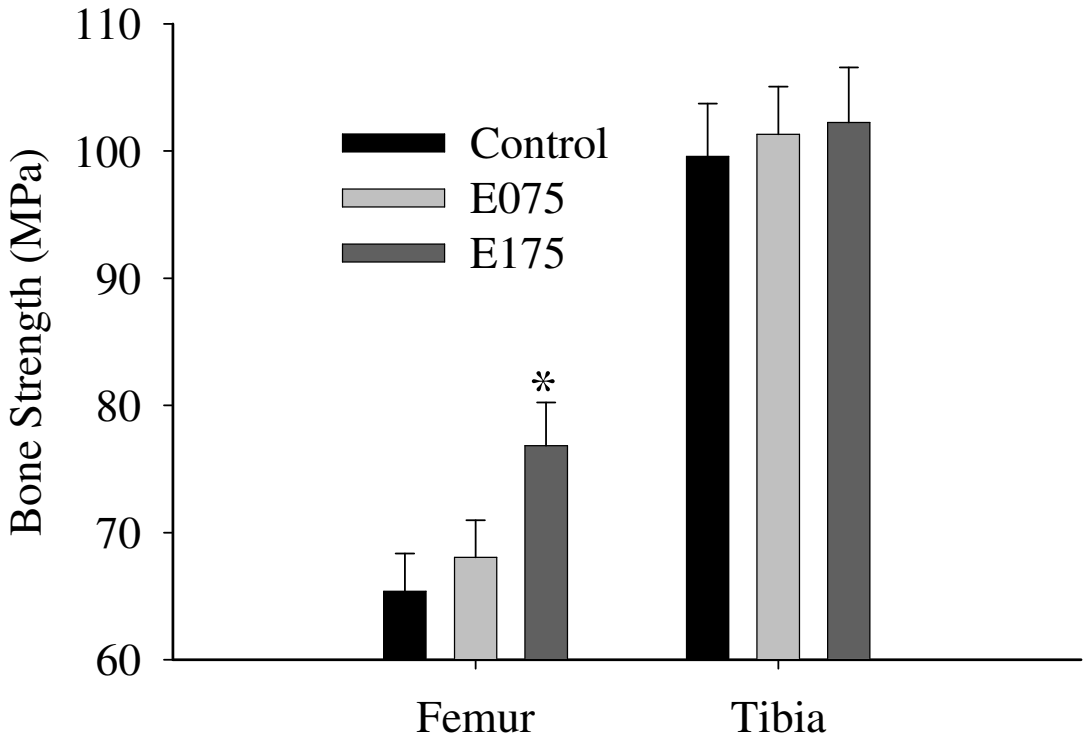

Figure A-42: Bone strength. First trimester maternal alcohol consumption altered fetal bone strength (MPa). The E175 (1.75 g/kg) group exhibited significantly higher (*) bone femoral bone strength compared with that in the control group whereas the tibial strength was not altered. The E075 (0.75 g/kg) was not altered. Alcohol improved fetal femoral strength (MPa) while tibial strength was not altered. This value was calculated as the material strength at the point of ultimate force as bone strength equal to FSD/8I, where F is the ultimate force, S is the span (4 cm), D is the anterior-posterior diameter, and I is the cross sectional moment of inertia.

APPENDIX B

Table B-1: Maternal P_aO_2 (mm Hg)

Time (min)	**Saline control** **P_aO_2 (mm Hg)**	**Alcohol** **P_aO_2 (mm Hg)**
0	100	98
30	97	99
60	102	97
90	98	103
120	100	96

Values measured from samples collected on days 90 and 132 of gestation were combined as they were not different. No statistically significant differences were found between alcohol and saline groups, and among different time points. Similarly no interaction between these two factors was revealed.

Table B-2: Maternal plasma amino acid concentrations

Amino acids	SC	Ethanol	Acidemia
ARG	142 ± 10	148± 13	204 ± 24*
GLU	106 ± 14	64 ± 8*	99 ± 20
ASP	8 ± 0	5 ± 1	8 ± 1
SER	62 ± 4	60 ± 8	85 ± 8*
THR	45 ± 4	52 ± 8	62 ± 10
CIT	119 ± 17	123 ± 15	145 ± 25
ALA	97 ± 7	77 ± 12	102 ± 4
TRP	20 ± 1	22 ± 1	27 ± 4
ASN	23 ± 1	26 ± 4	37 ± 3*
GLN	179 ± 16	112 ± 15*	165 ± 12
THR	45 ± 3	52 ± 8	62 ± 10
MET	13 ± 0.5	20 ± 2*	23 ± 2*
LEU	66 ± 7	84 ± 7*	83 ± 7*
HIS	22 ± 3	23 ± 1	23 ± 1
TAU	76 ± 12	62 ± 11	75 ± 18
TYR	36 ± 2	40 ± 3	48 ± 5
VAL	79 ± 3	148 ± 24*	170 ± 19*
PHE	29 ± 1	28 ± 0	32 ± 2
ILE	59 ± 3	66 ± 6	69 ± 3
ORN	35 ± 3	35 ± 4	44 ± 4
BCAA	204 ± 11	298 ± 35*	322 ± 22*

Maternal plasma amino acid concentrations (μmol/L) at the beginning of the last day of experiment were not different among groups for most amino acids. Glutamine and glutamate were significantly (*) reduced in the ethanol group; arginine, asparagine, serine were significantly elevated in the acidemic group, while valine, methionine, and total branched chain amino acids (BCAA) were significantly higher in the ethanol and the acidemic groups compared with that in the pair-fed saline control group (SC).

Table B-3: Treatment conditions and their acid-base status

#	Treatment	Maternal arterial oxygen status	Maternal acid base status	Fetal arterial oxygen status	Fetal acid-base status
1	Normal control	Not measured			
2	Pair-fed saline control	Normox	No change	No change	No Change
3	Ethanol	Mild transient reduction in P_aO_2	Acidemic	No change	Acidemic
4	Ethanol-Normox	No change	Acidemic	No change	Acidemic
5	Acidemic-Normox	No change	Acidemic	No change	Acidemic
6	Acidemic-hypox	Mild transient reduction in P_aO_2	Acidemic	No change	Acidemic
7	Ethanol-TI	Normox	No change	No change	No Change
8	Saline-TI	Normox	No change	No change	No Change

Nine treatment groups were used in this study: (1) an untreated normal control group, (2) a pair-fed saline control, (3) an ethanol group (produced fetal normoxemia and acidemia and a mild transient reduction in maternal P_aO_2), (4) an acidemic-normox group, (produced fetal and maternal normoxemia and acidemia), (5) an ethanol-normox group (produced fetal and maternal normoxemia and acidemia), (6) an acidemic-hypox group (produced fetal normoxemia and acidemia and a mild transient reduction in maternal P_aO_2), (7) an ethanol-TI group, and (8) a saline-TI group and (7) a fetal instrumented group, in order to measure fetal blood gases and acid-base status.

Table B-4: Fetal and maternal bone diameter (cm)

Group	Fetal Femur Diameter (cm)	Fetal Tibia Diameter (cm)	Maternal Femur Diameter (cm)	Maternal Tibia Diameter (cm)
NN	1.01 ± 0.02	0.93 ±0.02	2.23 ± 0.07	1.89 ± 0.07
SC	0.95 ± 0.03	0.93 ±0.02	2.33 ± 0.09	2.01 ± 0.06
E075	0.97 ± 0.01	0.91 ±0.01	2.28 ± 0.04	1.92 ± 0.05
E175	0.91 ± 0.07	0.85 ±0.06	2.33 ± 0.04	1.95 ± 0.02

NN: normal control group; SC: saline control group; E075, E175: alcohol dosage 0.75 and 1.75 g/kg of body weight respectively. Values are mean ± SEM. No significant differences were found among groups.

Table B-5: Fetal and maternal bone cross sectional moment of inertia (cm^4)

Group	Fetal Femur CSMI (cm^4)	Fetal Tibia CSMI (cm^4)	Maternal Femur CSMI (cm^4)	Maternal Tibia CSMI (cm^4)
NN	0.0456± 0.003	0.032 ± 0.002	0.79 ± 0.10	0.52 ± 0.07
SC	0.034 ± 0.003	0.036 ± 0.004	0.91 ± 0.12	0.66 ± 0.07
E075	0.038 ± 0.002	0.035 ± 0.002	0.88 ± 0.06	0.52 ± 0.05
E175	0.032 ± 0.007	0.036 ± 0.007	0.87 ± 0.03	0.54 ± 0.04

NN: normal control group; SC: saline control group; E075, E175: alcohol dosage 0.75 and 1.75 g/kg of body weight respectively. Values are mean ± SEM. No significant differences were found among groups.

Table B-6: Maternal bone Strength (MPa) and length (cm)

Group	Maternal Femur Strength (MPa)	Maternal Tibial Strength (MPa)	Maternal Femur Length (cm)	Maternal Tibia Length (cm)
NN	53.2 ± 7.2	80.8 ± 3.2	20.4 ± 0.6	23.4 ± 0.7
SC	53.0 ± 3.0	75.5 ± 4.9	21.3 ± 0.6	24.6 ± 0.7
E075	44.2 ± 3.3	87.5 ± 6.6	20.6 ± 0.4	23.8 ± 0.4
E175	46.4 ± 3.7	88.7 ± 4.6	21.5 ± 0.3	24.5 ± 0.3

NN: normal control group; SC: saline control group; E075, E175: alcohol dosage 0.75 and 1.75 g/kg of body weight respectively. Values are mean ± SEM. No significant differences were found among groups.

VITA

Jayanth Ramadoss
10310 Willow Brook Drive
College Station, TX 77845
E-mail: jramadoss@cvm.tamu.edu

Education

- Ph.D., Biomedical Sciences, Texas A&M University, College Station, May 2008.
- M.Sc. (Hons.) Chemistry, Birla Institute of Technology & Science, Pilani, India, Aug 2002.
- B.E.(Hons.) Electronics & Instrumentation, Birla Institute of Technology & Science, Pilani, India, Aug 2002.

Work Experience

- Graduate research assistant, Texas A&M University, College Station (1/2004-Present).
- Teaching assistant, Texas A&M University, College Station (1/2005-Present).
- Graduate tutor, Syracuse University, NY (8/2003-12/2004).
- Systems engineer, Tata Consultancy Services Ltd., Mumbai, India (8/2002-7/2003).
- Software engineer trainee, Honeywell Inc., Bangalore, India (1/2002-6/2002).
- Academic counseling board student instructor, BITS Pilani, India (8/2000-5/2002).
- Summer research student, Indian Institute of Science, Bangalore, India (5/2000-7/2000).

Honors & Awards

- Outstanding graduate student award for excellence in academics and research, Texas A&M University, College Station, TX (3/2007).
- Selected to present at the FASt satellite session (7/2007).
- Research travel award, Texas A&M University, College Station, TX (6/ 2007).
- Research travel award, Texas A&M University, College Station, TX (6/2006).
- Phi Kappa Phi Honors Society (2/2006).
- Research travel award, Texas A&M University, College Station, TX (3/2005).
- Research grant award from the Office of Graduate Studies, Texas A&M University, College station, TX (3/ 2005).
- Award for Academic Excellence sponsored by NIIT, India (8/1997).