MATERNAL ADRENOCORTICOTROPIN, CORTISOL AND THYROID HORMONE RESPONSES TO CHRONIC BINGE ALCOHOL EXPOSURE THROUGHOUT GESTATION: OVINE MODEL

A Thesis

by

URSULA TRESS

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

MASTER OF SCIENCE

December 2007

Major Subject: Veterinary Physiology

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Approved by:

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December 2007

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ABSTRACT

Maternal Adrenocorticotropin, Cortisol and Thyroid Hormone Responses to Chronic Binge Alcohol Exposure Throughout Gestation: Ovine Model.

(December 2007)

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This study investigated the effect of chronic alcohol exposure on the responses of the maternal hypothalamus-pituitary adrenal axis (HPA-axis) and thyroid hormones throughout gestation using an ovine model. Maternal plasma concentrations of ACTH, cortisol and the thyroid hormones T_3, free T_4 and total T_4 were determined in response to infusion of 0.75, 1.25 and 1.75 g/kg alcohol.

Maternal endocrine responses to alcohol administration have been investigated before in rodent models. However, this is the first study using a large animal model (sheep), in which all three human trimester equivalents occur in utero. Different concentrations of alcohol were administered intermittently from gestational day 4 to 132 in a pattern that modeled human binge drinking during pregnancy. Maternal blood samples were collected on specific days (GD 6, 40, 90, 132) and at multiple time-points (0, 0.5, 1, 1.5, 2, 6, 24 hours) and were analyzed to determine blood alcohol concentrations (BACs) and ACTH, cortisol, free T_4, total T_4 and T_3 plasma concentrations.

Alcohol readily permeates the placenta and can directly affect fetal cells and tissues. Alcohol also causes endocrine imbalances in the mother and interferes with maternal-fetal hormonal interactions and the mother's ability to maintain a healthy pregnancy, thus also indirectly affecting fetal development. Sheep receiving either 0.75, 1.25 or 1.75 g/kg alcohol achieved peak BAC values of 93 $\pm$ 5, 126 $\pm$ 5 and 183 $\pm$ 5 respectively. Alcohol exposure resulted in increased plasma ACTH and cortisol concentrations peaking at 2 hours after beginning of the infusion and returning to baseline values at 6 hours after beginning of the infusion. There was no effect of alcohol on any of the plasma thyroid hormone concentrations. Thyroid hormone concentrations changed as a result of progressing pregnancy. Plasma concentrations of total T_4 and free T_4 were higher on gestational days 6 and 40 compared to GDs 90 and 132, and plasma T_3 concentrations were highest on GD 6.

The results of this study show that alcohol stimulates the HPA-axis in a dose dependent fashion in pregnant sheep. The response of the HPA-axis to repeated alcohol exposure throughout gestation remained unchanged. Alcohol exposure did not affect the release of thyroid hormones. Thyroid hormone concentrations changed during pregnancy in sheep in a manner similar to changes observed in pregnant women.

DEDICATION

This work is dedicated to my wonderful mother Renate Tress

ACKNOWLEDGEMENTS

I would like to thank my committee chair, Dr. Timothy A. Cudd, and my committee members, Dr. Wei-Jung Chen, Dr. Yanan Tian, and Dr. Jeremy S. Wasser, for their guidance and support throughout the course of this research.

Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience.

NOMENCLATURE

FAS	Fetal Alcohol Syndrome
FASD	Fetal Alcohol Spectrum Disorder
ARBD	Alcohol-Related Birth Defects
ARND	Alcohol-Related Neurodevelopmental Disorder
HPA-axis	Hypothalamus-Pituitary Adrenal Axis
HP-axis	Hypothalamus-Pituitary Axis
CRF	Corticotropin Releasing Factor
ACTH	Adrenocorticotropin
GR	Glucocorticoidreceptor
T_4	3, 5, 3', 5'-Tetraiodothyronine
T_3	3, 5, 3'—Triiodothyronine
BAC	Blood Alcohol Concentration in mg/dl
GD	Gestational Day

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CHAPTER I

INTRODUCTION: IMPORTANCE OF RESEARCH

Alcohol is well known for its teratogenic effects, causing fetal alcohol syndrome (FAS), a condition considered to be the leading known preventable cause for mental retardation in the Western world (Abel and Sokol, 1991). Considerable efforts have been undertaken to educate women about the consequences of alcohol consumption during pregnancy, nevertheless some women continue to drink and the incidence of FAS has not declined significantly since (Caetano et al., 2006). This has led to numerous investigations studying the mechanisms by which alcohol may cause its detrimental effects. A more detailed knowledge about the specific mechanisms by which alcohol causes adverse consequences during gestation is necessary to development of early treatments or interventions designed to prevent or alleviate FAS. The current study investigates maternal endocrine responses to alcohol administered to pregnant sheep throughout all three trimesters.

Several studies have shown that alcohol can disturb the endocrine homeostasis and the HPA-axis appears to be highly susceptible (Zhang, 2005; Cudd, 2001; 2002). If such disturbances occur during pregnancy, the maternal-fetal hormonal interactions are likely to be affected. Both thyroid hormone and cortisol in appropriate concentrations are critical for normal fetal brain and somatic development and maternal contributions of both hormones are essential (de Escobar et al., 2004b; Fowden and Forhead, 2004; Koibuchi and Chin, 2000).

This thesis follows the format of *Alcohol*.

Similarities in brain defects between children prenatally exposed to alcohol or to high glucocorticoid levels have been reported, suggesting that alcohol induced HPA-axis activation may be contributing to the neuroanatomical and neurobehavioral deficits attributed to prenatal alcohol exposure (Huang et al., 1999; Kim et al., 1999; Weinberg, 1989). Gestational overexposure to glucocorticoids can result in hypertension, glucose intolerance, and permanent alteration of the HPA-axis function leading to hyperresponsiveness to stress and elevated basal glucocorticoid levels later in life (Zhang et al., 2005; Seckl, 2004; Matthews, 2000). Furthermore, glucocorticoids appear to increase the vulnerability of the brain to adverse events such as neurotoxins or psychosocial stress (Gubba et al., 2000).

Hypothyroidism can affect maturation of the cerebellar cortex in rats in ways similar to prenatal alcohol exposure (Kornguth et al., 1979). Children born to hypothyroid mothers or with congenital hypothyroidism may show similar behavioral and neurological abnormalities compared to children with FAS (Man and Serunian, 1976). Fetal hypothyroidism leads to intrauterine growth retardation and altered development of the nervous system (Fowden and Forhead, 2004; Zoeller et al., 2002). It is known that thyroid hormone responses are abnormal in adult humans and rats chronically exposed to ethanol (Herman et al., 2002; Baumgartner et al., 1994; Cicero, 1981; Portoles et al. 1985).

ACTH, cortisol and thyroid hormone secretions have been shown to be altered in adult and fetal animals after alcohol consumption (Cudd et al., 2001; Cudd et al., 2002; Iqbal et al., 2005; Ogilvie et al., 1997). Therefore, it is possible that ethanol induced

changes in plasma ACTH, cortisol and thyroid hormone levels are contributing factors leading to alcohol related birth defects (ARBD). A common characteristic among different species is that the HPA-axis matures during fetal life, very late in gestation (Challis, et al. 2001). Plasma concentrations of cortisol in ovine fetuses are significantly lower compared to maternal values and the maternal adrenal gland is the major source for fetal cortisol until about 47-26 days before term. Later in pregnancy, about 25-12 days before term, the contribution of the fetal adrenal gland to fetal plasma cortisol increases up to one third of the total, until all of the fetal cortisol is supplied by the fetal adrenals 1-2 weeks before the end of gestation (Hennessy et al., 1982; Rose et al., 1982).

Thyroid hormones can be detected in placental, embryonic and fetal tissues long before the onset of fetal thyroid hormone production. These hormones are of maternal origin and the concentration in fetal tissues is directly proportional to maternal concentrations until the fetal thyroid gland becomes functional at the end of the first trimester and contributes about 25-50% of the total circulating fetal thyroid hormone supply (de Escobar et al., 2004a,b; Calvo et al., 2002).

Therefore, the measurement of maternal ACTH, cortisol, and thyroid hormones might give valuable information about the fetal concentrations of these hormones.

The goal of this study was to monitor maternal responses of the HPA-axis and thyroid gland to intermittent exposure to different concentrations of alcohol throughout pregnancy as potential indicators of abnormal fetal concentrations of these hormones possibly contributing to ARBD.

FETAL ALCOHOL SYNDROME (FAS)

Children born to mothers, who abused alcohol during their pregnancy, are at a great risk to give birth to children displaying a characteristic pattern of permanent birth defects. The symptoms associated with fetal alcohol exposure were defined by Jones et al. as fetal alcohol syndrome (FAS) and were very similar to those reported reported by Lemoine at al. in a French publication several years earlier (Lemoine et al., 1968; Jones et al., 1973). Since then considerable research has been performed to characterize children affected by FAS.

Historically, the diagnosis of FAS requires four characteristics:

1) Evidence of maternal alcohol consumption during pregnancy

2) A distinctive pattern of facial abnormalities, (smooth philtrum, thin upper lip, short palpebral fissures)

3) Pre- and or postnatal growth retardation and

4) Evidence of central nervous system dysfunction

(NIAAA, Alcohol Alert, No. 50, 2000; Riley and McGee, 2005).

Figure 1 depicts the cardinal facial features observed in a young child affected with FAS.

More recently the term "Fetal Alcohol Spectrum Disorder" (FASD) has emerged as umbrella term to encompass the wide variability in type and severity of damage that can occur in children whose mothers abused alcohol during pregnancy. FASD is not used as a diagnostic term; rather it describes the full range of disabilities that may result from prenatal alcohol exposure including FAS, and partial expressions of FAS such as "Alcohol-Related Neurodevelopmental Disorder (ARND)" and "Alcohol-Related Birth

Defects (ARBD)". These outcomes may be physical, mental or behavioral and possibly lifelong (Riley and McGee, 2005). Although tremendous efforts have been made to educate women about the adverse effects of alcohol consumption on the developing fetus, FAS nonetheless remains the leading known preventable cause for mental retardation in the western world and constitutes a costly major public health problem (Abel and Sokol, 1991; Riley and McGee, 2005). It is estimated that the prevalence of FAS in the USA alone lies between 0.2 and 2.0 cases per 1,000 live births. This rate is comparable or even higher than the prevalence of other developmental disabilities such as "Down Syndrome" or "Spina Bifida" (CDC, 2004). The consequences of FAS not only affect individuals and family members, but also the community and society in general. The economic impact of FAS is substantial and the estimated lifetime medical and social costs of each child with FAS are as high as US$800,000 (Bloss, 1994). FAS is not restricted to certain ethnicities or populations, and occurs throughout the worldalthough certain populations seem be more affected than others (Riley and McGee, 2005). There is an ongoing need to educate women about the potential risks associated with drinking during pregnancy, but it is also clear that more research is necessary to evaluate and develop possible strategies and treatments to prevent or ameliorate the effects of prenatal exposure to alcohol. A challenging aspect of FAS faced by physicians is to come to a diagnosis as early as possible. Women who drink during pregnancy may not correctly report about the amount, timing, and pattern of their alcohol consumption to their physician. Diagnosis of FAS relies on the identification of the aforementioned four cardinal features. Ongoing research investigating the mechanisms by which alcohol

leads to the known effects may also be useful for developing and refiningmethods for an explicit, objective and early diagnosis that is less dependent upon physical features or maternal self-report.

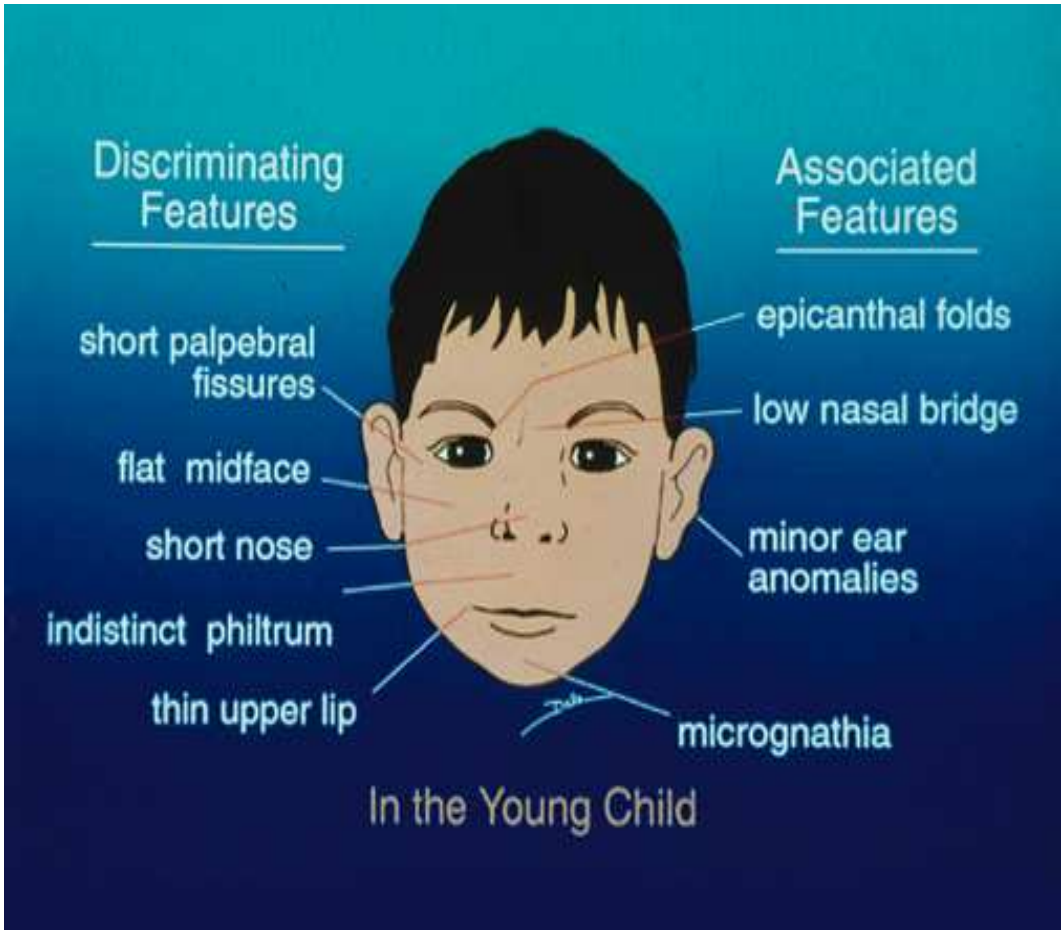

Figure 1: Distinctive Pattern of Facial Features of FAS in the Young Child

Streissguth, A.P., and Little, R.E., "Alcohol, Pregnancy, and the Fetal Alcohol Syndrome," 2nd Edition. Unit 5 of "Alcohol Use and Its Medical Consequences. A Comprehensive Slide Teaching Program for Biomedical Education." Developed by Project Cash of the Dartmouth Medical School.

SHEEP MODEL

Because of ethical considerations and experimental difficulties in studying the effects of alcohol in humans, animal models have been developed and are widely used in various aspects of alcohol research, such as alcohol dependence and alcohol effects. A crucial advantage of utilizing animal models lies in the ability to control several factors, such as amount, timing, duration and route of alcohol administration, shown to be important determinants of alcohol effects (Tabakoff and Hoffman, 2000). Animal models currently used in alcohol research include non-human primates, rodents (mice, rats, guinea pigs), sheep, pigs, fish, insects and round worms (Cudd, 2005). Depending on the research hypothesis, certain animal models are better suited than others.

For example, it is important to consider the differences in brain development between humans and different species intended to be used as animal model. The maximum velocity of human brain growth occurs in utero at the time of parturition, whereas in rats and mice it occurs postnatally (Cudd, 2005). Sheep have been widely used in studies investigating endocrine responses (Keller-Wood et al., 1998). The advantages of using sheep are the large size of the fetus and its accessibility and robustness permits successful surgical instrumentations (Keller-Wood et al., 1998). Furthermore, the sheep has been extensively used as experimental model for human pregnancy, because similar to humans it possesses a long gestation (~147 days) and all three human trimester equivalents of the sheep fetus occur in utero (Ruiz et al., 1972; Cudd, 2005). A plethora of information about the ovine maternal and fetal physiology especially with regard to adrenal gland function is readily available (Liggins, 1974; Bell

at al., 1991; Nolten and Ruekert, 1981). This was of particular benefit for our study, as we were interested in the responses of the hypothalamic-pituitary adrenal gland axis (HPA-axis) and thyroid gland to alcohol exposure.

HYPOTHALAMUS-PITUITARY ADRENAL AXIS (HPA-AXIS)

The hypothalamus-pituitary-axis is a complex and dominant component of the neuroendocrine system and regulates the function of the thyroid, adrenal, and reproductive glands (Genuth, 1998a). The hypothalamus is the key regulator of pituitary function, receiving and integrating input from various areas of the brain and channeling them to the pituitary gland. Signals such as pain, stress, fear, temperature, etc. influence pituitary function. The pituitary gland consists of an anterior and posterior part. The posterior pituitary or neurohypophysis secretes antidiuretic hormone (ADH or vasopressin) and oxytocin and the anterior pituitary or adenohypophysis secretes a group of tropic hormones: adrenocorticotropic hormone (ACTH, adrenocorticotropin), thyroid-stimulating hormone (TSH), luteinizing hormone (LH), follicle-stimulating hormone (FSH), growth hormone (GH) and prolactin. Releasing or inhibiting factors from the hypothalamus control the release of the tropic hormones from the adenohypophysis, which then act on their respective target glands in the periphery. Feedback mechanisms at several levels control hormone concentrations in the circulation.

Corticotropin releasing factor (CRF) from the paraventricular nucleus of the hypothalamus leads to the secretion of ACTH from the anterior pituitary. Adrenocorticotropin is a peptide hormone that regulates growth and maturation of the

adrenal cortex and synthesis and secretion of its steroid hormones. The stimulation of the release of glucocorticoids from the adrenal cortex is an essential function of ACTH. The predominant glucocorticoid in humans and most mammals is cortisol (rodents release corticosterone). ACTH secretion displays a marked diurnal pattern with peak secretion about 2-4 hours before awakening. ACTH induced cortisol secretion from the adrenal cortex is closely linked to the pattern of ACTH secretion following with a delay of about 10-30 minutes. Cortisol inhibits further ACTH secretion via negative feedback mechanism. Cortisol is necessary for life. It maintains the availability of glucose, supports responsiveness of the vasculature, modulates the function of the central nervous system and has a profound affect on the immune system and inflammatory responses (a, 1998). Cortisol can freely enter target cells and penetrate the blood brain barrier. Most of the cortisol effects are mediated by one of two types of glucocorticoid receptors (GR 1 and 2). The receptors when bound enter the nucleus and activate or repress the transcription of certain genes. Type 2 GR is mostly found in peripheral tissues and GR 1 mediates cortisol effects specific to the brain, especially the hippocampus (de Kloet et al., 1998). The glucocorticoids cortisol and corticosterone play a major role in the preparation of the fetus for extrauterine life. Cortisol promotes cellular differentiation and induces a wide range of enzymes that are necessary after birth. In most species, especially when the newborn is precocious and must be self-sufficient to survive such as the lamb, cortisol in the fetal circulation rises sharply towards the end of gestation (Liggins, 1994). The HPA-axis is also important for coordinating readiness for birth with the timing of birth (Wood, 2005). An increase in the activity of the fetal HPA-axis in

sheep triggers parturition in this species (Liggins et al., 1973; Challis and Brooks, 1989). In sheep, the increase of ACTH and cortisol secretion seems to be caused by maturation of the HPA-axis combined with reduced feedback effects of cortisol just before term (Wood, 1988; Keller-Wood and Wood, 2001). Fetal adrenal maturation begins at around 130 days of gestation and progresses until term, resulting in an exponential increase in fetal plasma cortisol concentrations, which signals the readiness of the fetus for birth and triggers parturition. Excessive glucocorticoid exposure during gestation has been reported to negatively affect the weight of individual fetal tissues and to result in overall growth retardation (Challis, et al. 2001).

THYROID HORMONES

The hormones synthesized and secreted by the thyroid gland are 3, 5, 3', 5'-tetraiodothyronine (thyroxine or T_4) and 3, 5, 3'—triiodothyronine (T_3). Thyroxine (T_4) is the predominant product (~90%) and the biologically active form is T_3, which is formed from T_4. Synthesis and release of the thyroid hormones are under the control of the hypothalamus-pituitary axis. Thyrotropin releasing hormone (TRH) from the hypothalamus controls the release of thyrotropin stimulating hormone (TSH) from the anterior pituitary. Thyrotropin stimulating hormone promotes synthesis and release of the thyroid hormones by the thyroid gland. In order to form the biologically active T_3, the almost inactive T_4 must undergo enzymatic activation by 5'deiodinases present in different tissues (Larsen et al., 1998). Most of the thyroid hormones in plasma are protein bound. Aside from their important role in regulation of the overall rate of body

metabolism, thyroid hormones are required for normal brain development during fetal life (de Escobar, 2004a,b). Thyroid hormones act by binding to nuclear receptors and modulating the transcription of responsive genes. Fetal thyroid hormone receptors are widely distributed in the brain prior to the onset of fetal thyroid hormone production, and the fetus depends solely on maternal thyroid hormone transfer during this early time period of development. Studies in several species have shown that there is indeed a substantial transfer of maternal thyroid hormones across the placenta until the fetus acquires the ability to synthesize thyroid hormones at the end of the first trimester and contributes to the thyroid hormone supply (de Escobar et al., 2004a; Calvo et al., 2002). Furthermore, the placenta contains deiodinases that convert T_4 into the biologically active form T_3. Insufficient amounts of thyroid hormones cause abnormal development of almost every organ system resulting in the syndrome “cretinism” (Oppenheimer and Schwartz, 1983; Delange, 1996). The developing brain is especially affected by severe functional deficits leading to mental retardation, ataxia, spasticity, and deafness (DeLong, 1996).

CHAPTER II

MATERIALS AND METHODS

ANIMALS AND BREEDING

All procedures involving animals that were employed in this study were approved by the University Laboratory Animal Care Committee at Texas A&M University. Suffolk ewes (n=31) were maintained on Bermuda grass pasture and supplemented with alfalfa. Pregnancies of known conception date were achieved by manipulating the estrous cycle through the use of progesterone impregnated vaginal implants (EAZI-BREED™ CIDR®, Pharmacia & Upjohn Ltd., Auckland, New Zealand). Implants were inserted then removed 11 days later at which time intramuscular prostaglandin $F_{2\alpha}$ (20 mg LUTALYSE®, Pharmacia & Upjohn, Kalamazoo, MI) was 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 gestational day four, saline and alcohol group subjects were moved into individual pens. From these pens, the ewes were able to maintain visual contact at all times with herdmates in adjacent pens. Conditions of constant temperature (22° C) and a fixed light dark cycle (12:12) were maintained. Once confined, the saline and alcohol treatment group subjects received two kg of complete pellet ration daily (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 (figure 2) remained in pens with herdmates throughout the study and were offered, as a group, an equivalent amount of

feed compared to subjects in the saline and alcohol control groups, though individual feed consumption was not monitored.

Figure 2: Normal Control Sheep

SURGERY

On gestational day four, an intravenous catheter (16 gauge, 5.25 in Angiocath™ Becton Dickinson, Sandy, UT) was placed percutaneously into the jugular vein for the administration of the infusions and the collection of blood samples. On gestational day 42 after confirmation of pregnancy ultrasonographically, ewes underwent surgery to chronically implant vascular access ports (V-A-P™, Model CP47P, Access Technologies, Skokie, IL). The ewes were not subjected to surgery and general anesthesia until after the first trimester to avoid early embryonic losses. In brief, anesthesia was induced with diazepam (0.2 mg/kg intravenously, Abbott Laboratories, North Chicago, IL) and ketamine (0.4 mg/kg intravenously, Ketaset®, Fort Doge, IA). The ewes were intubated and 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. The catheters were advanced into the aorta and vena cava via the femoral artery and vein respectively. After completion of surgery, ewes received flunixin meglumine (1.1 mg/kg intramuscularly, Banamine®, Scherring-Plough, Union, NJ), a prostaglandin synthase inhibitor to alleviate postoperative pain. To prevent infections postoperative antibiotics were administered twice daily for five consecutive days (25 mg/kg subcutaneously, ampicillin trihydrate, Polyflex®, Aveco, Fort Dodge, IA, and 2mg/kg intramuscularly, gentamicin sulfate, Gentavet®, Velco, St.Louis). Sheep entered the study at least five days after surgery. Treatment infusions were given through the venous port and blood samples were collected from the arterial port.

ALCOHOL TREATMENT PROTOCOL

Animals in the alcohol treatment groups (0.75, 1.25 or 1.75 g/kg alcohol) and the saline control group received intravenous infusion delivered by peristaltic pump (Masterflex, model 7014-20, Cole Parmer, Niles, IL) through a 0.2 μm bacteriostatic filter shown in figures 3 and 4. Pumps were calibrated each time before infusion. Alcohol infusions were 40% W/V in sterile saline (0.9% Sodium Chloride, Injection, USP, Hospira, Lake Forest, IL) administered at the same rate over one hour. Treatments began on gestational day four and were administered on three consecutive days per week, followed by four consecutive days without alcohol mimicking a binge drinking paradigm. This pattern of infusions was continued until the final alcohol exposure on gestational day 132. As on any other day, on experimental days investigators entered the room housing the sheep at 0800 hr to feed the sheep and monitor their food consumption. The ewes were prepared to receive their respective infusion and to collect blood samples by cleaning and disinfecting the skin covering the vascular access ports.

After each collection of blood, the arterial catheter was flushed with five cc of 0.9% saline and three cc of 0.1% Heparin (Heparin, sodium injection, USP, 5000 USP units/1 ml, Baxter Healthcare Corporation, Deerfield, IL) in 0.9% saline. After the collection of the last blood sample, the catheters were flushed with five cc 0.9% saline and 0.4 cc Heparin in 2.6 cc saline to prevent the formation of blood clots.

The collection of a blood sample during the experiment is presented in figure 5. The peristaltic pumps for delivery of the infusions were calibrated each time immediately before use. Infusions were delivered continuously at the same rate over 60 min from 0830-0930 hr. Blood was obtained from the jugular vein catheter on gestational day 6 and gestational day 40 and from the femoral arterial catheter on gestational day 90 and gestational day 132. The first blood sample for the measurement of blood alcohol concentrations (BAC) and plasma hormone concentrations was collected immediately prior to the commencement of the infusions (0 minute) and then every 30 min for 2 hrs and at 6 and 24 hrs. On gestational day 133, the sheep were euthanized using an overdose of pentobarbital. The fetuses were removed and fetal organs were collected and weighed.

Figure 3: Sheep Infusion Right Side.

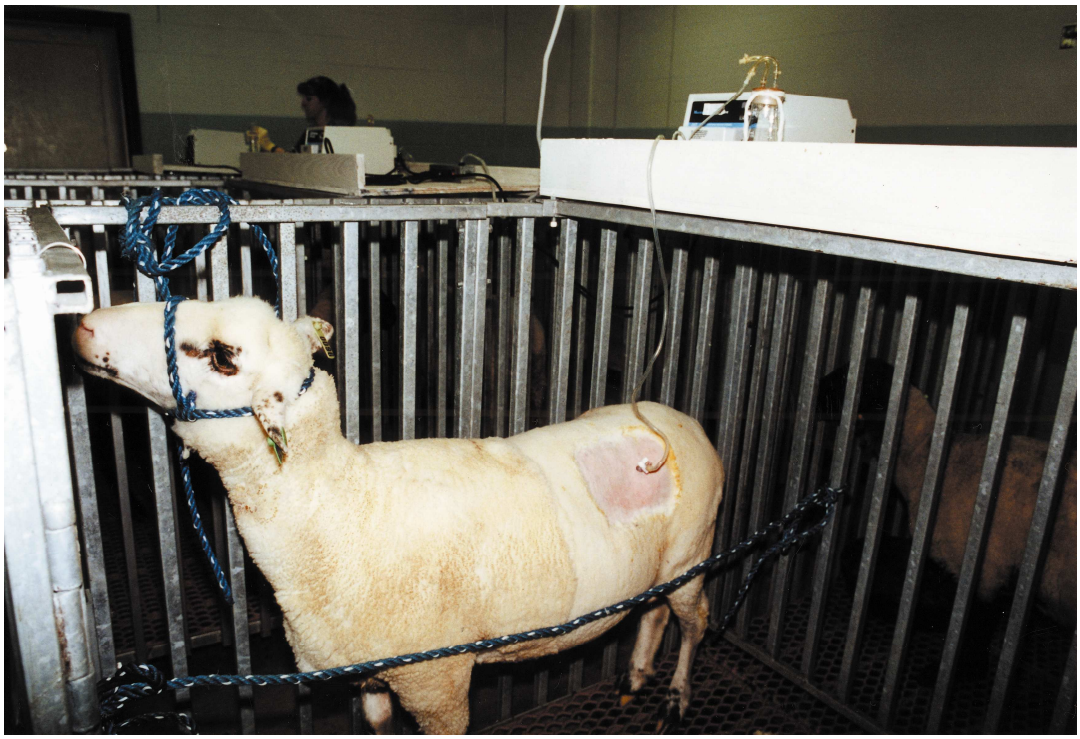

Figure 4: Sheep Infusion Left Side.

Figure 5: Collection of Blood Samples

ASSAY METHODOLOGIES

For the measurement of blood alcohol concentrations (BACs) 20 µl aliquots of blood were collected into microcapillary tubes and transferred into vials containing 0.6 N perchloric acid and 4 mM of n-propyl alcohol (an internal standard) in distilled water. The vials were tightly capped with a septum sealed lid and stored at room temperature for at least 24 hrs before being analyzed by headspace gas chromatography (Varian Associates, Model 3900, Palo Alto, CA). The vapor above the liquid was injected into the gas chromatograph. The basic 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). Blood samples for hormone analyses (4 ml) were collected and placed in chilled polystyrene tubes containing 200 µl of 0.5 M ethylenediaminetetraacedic acid (EDTA). Samples were collected every 30 min for 2 hrs, beginning immediately before the administration of infusions, and then at 6 and 24 hrs. The tubes were maintained in icewater until being centrifuged for 20 min at 2800 x g at 4°C. Plasma was separated and stored in aliquots at -20°C. Plasma ACTH and cortisol concentrations were measured by specific radioimmunoassays. ACTH was measured using a commercial radioimmunoassay (DiaSorin Incstar, Stillwater, MN) validated in sheep plasma. The coefficients of variation for intra- and interassay variability were 6.4% and 8.8% respectively (Cudd et al., 2001). Cortisol was measured by specific radioimmunoassay (Cortisol Coat-a-Count™, Diagnostic Products Corp., Los Angeles, CA). The assay was modified by the addition of three standards (1.0 ng/µl, 2.5 ng/µl, and 5.0 ng/µl) to increase the accuracy

of low cortisol measurements. The coefficients of variation for intra- and interassay variability were 5.6% and 6.7% respectively (Cudd et al., 2001). Plasma total T_3, total T_4 and free T_4 concentrations were measured by specific radioimmunoassays (Total T_3 Coat-a-Count™, Total T_4 Coat-a-Count™, Free T_4 Coat-a-Count™ respectively, Diagnostic Products Corp., Los Angeles, CA) (Cudd et al., 2002). The T_4 assay has been validated for use in sheep (Moenter et al., 1991).

DATA ANALYSIS

Plasma ACTH and cortisol were measured at all seven time points on each of the four different GDs, while thyroid hormones were measured only at time 0 on each of the five different GDs. Mixed analysis of variance was used to analyze the ACTH and cortisol data, with GD and time point as within factors and treatment as between factor.

T_3, T_4, and free T_4 data were analyzed using a mixed ANOVA with treatment as between and GD as within factor. Student-Newman-Keuls test was used for post-hoc tests.

The weights of the fetal organs were subjected to one way ANOVAs with alcohol dose as the factor.

In all cases statistical significance was defined as a p-value ≤ 0.05 data are presented as the mean $\pm$ 1 SEM.

CHAPTER III

RESULTS

The mean blood alcohol concentrations (BACs) measured on gestational days 6, 40, 90 and 132 for the animals in the 0.75 g/kg, 1.25 g/kg and 1.75 g/kg alcohol groups peaked at 1 hr which coincided with the end of the infusion period and were 93 ± 5 mg/dl, 126 ± 5 mg/dl and 183 ± 5 mg/dl respectively. There was no significant difference between the BACs on the different gestational days within one alcohol group and therefore the data were combined.

Maternal plasma ACTH analyzed by mixed ANOVA with time point and GD as within factors and treatment as between factor did not show a significant three way interaction. Correspondingly, there was no interaction between GD and time point, GD and treatment, or main effect of GD. There was however, a significant interaction between treatment and time point ($F_{18,574} = 2.088$, $p = 0.005$). There was also a main effect of treatment ($F_{3,574} = 5.803$, $p < 0.001$) and time point ($F_{6,574} = 7.911$, $p < 0.001$).

Post-hoc analysis revealed that the infusion of 1.75 g/kg alcohol resulted in increased ACTH plasma concentrations at 1 and 1.5 hours after the beginning of the infusion compared to all other treatment groups except the 1.25 g/kg alcohol group. At 2 hours after the beginning of the infusions ACTH plasma concentrations in the 1.75 g/kg alcohol group were higher than in all other treatment groups (figure 6).

As with ACTH, maternal plasma cortisol concentrations did not show a significant three-way interaction. There was also no interaction between GD and time point, GD and treatment, or main effect of GD. There was however, a significant

interaction between treatment and time point ($F_{18,588} = 6.520$, $p < 0.001$). Both, treatment ($F_{3,588} = 44.606$, $p < 0.001$) and time point ($F_{6,588} = 12.427$, $p < 0.001$) had main effects. Post-hoc analysis identified a significant elevation of plasma cortisol concentrations in the 1.75 g/kg alcohol dose group ($p < 0.001$) at 1, 1.5 and 2 hours after the beginning of the infusions compared to all other treatment groups (figure 7).

Thyroid hormone concentrations of the samples collected immediately before commencement of the infusion on all four GDs were analyzed by mixed ANOVA with GD as within factor and treatment as between factor. Gestational day was a significant main effect for the concentration of plasma T_3 ($F_{3,82} = 3.72$, $p = 0.0150$) but there was no main effect of treatment ($F = 0.56$, $p = 0.2005$) and no interaction between GD and treatment ($F = 0.82$, $p = 0.5833$). Plasma T_3 concentrations were significantly higher ($p < 0.05$) on GD 6 compared to all other gestational days (figure 8). Correspondingly, GD ($F_{3,82} = 10.11$, $p < 0.001$) was a significant main effect for total T_4, but neither were treatment, nor interaction between GD and treatment. Plasma total T_4 was significantly lower ($F = 11.349$, $p < 0.001$) on GDs 90 and 132 compared to GDs 6 and 40 (figure 8). For free T_4, there was a significant effect of GD ($F_{3,82} = 10.05$, $p < 0.001$) but not treatment, and there was no interaction between GD and alcohol dose. Free T_4 was lower ($p < 0.05$) on GDs 90 and 132 compared to GDs 6 and 40(figure 8).

There was no significant difference of the fetal organ weights when comparing between treatment groups (Table 1).

Table 1: Fetal Organ Weights
This table lists the weights of the fetal organs in gram as measured an gestational day 133 in the normal control group, pair fed saline control group, and the different alcohol groups (dosages: 0.75 g/kg, 1.25 g/kg, 1.75 g/kg). There was no significant difference among the fetal organ weights between the different groups.

	Normal Control	**Saline Control**	**Alcohol 0.75 g/kg**	**Alcohol 1.25 g/kg**	**Alcohol 1.75 g/kg**
Adrenals	0.42 ± 0.03	0.38 ± 0.04	0.40 ± 0.02	0.39 ± 0.06	0.39 ± 0.06
Thymus	5.95 ± 0.60	5.40 ± 0.70	5.08 ± 0.36	4.63 ± 1.3	5.44 ± 1.05
Thyroids	0.93 ± 0.13	0.87 ± 0.10	0.87 ± 0.10	0.96 ± 0.10	1.04 ± 0.08
Liver	106.73 ± 7.29	109.74 ± 10.97	107.84 ± 7.64	114.28 ± 13.27	122.98 ± 14.20
Spleen	5.69 ± 0.41	5.94 ± 0.85	5.09 ± 0.31	5.52 ± 0.66	5.66 ± 0.62
Heart	27.29 ± 2.64	28.44 ± 1.82	27.09 ± 1.92	29.07 ± 3.39	29.37 ± 2.77
Lungs	130.57 ± 7.82	134.90 ± 8.65	119.25 ± 8.13	117.69 ± 18.83	122.81 ± 14.69
Kidneys	21.51 ± 1.10	22.46 ± 1.15	22.00 ± 0.72	22.93 ± 2.35	21.47 ± 1.80

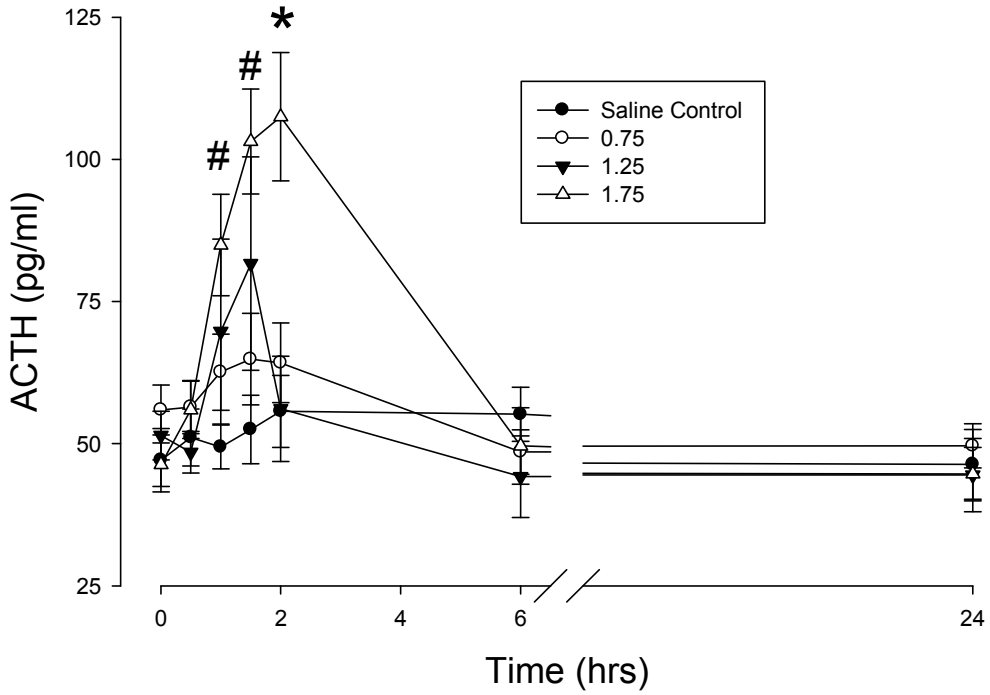

Figure 6: Maternal Plasma ACTH Concentrations over Time
This figure shows maternal plasma ACTH concentrations in pg/ml $\pm$ SEM. Maternal plasma ACTH concentrations (pg/ml) were measured from the beginning of the infusion period (0 min) every 30 min for 2 hours and at 6 and 24 hours on gestational days 6, 40, 90 and 132. ACTH concentrations in sheep within one treatment group were not different when compared between gestational days (not presented). Therefore, gestational day values were combined and treatment group responses over time are presented here. Maternal plasma ACTH concentrations at 1 hr and 1.5 hrs were significantly greater in sheep receiving 1.75 g/kg alcohol compared to all other groups except the 1.25 g/kg group(#) and compared to all other treatment groups at 2 hours (*) .

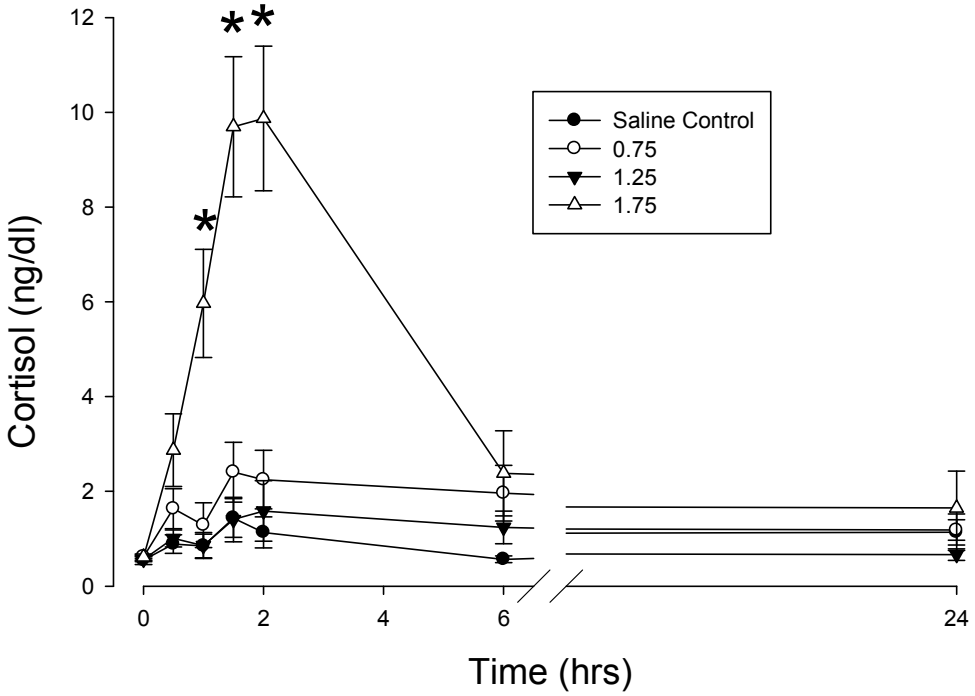

Figure 7: Maternal Plasma Cortisol Concentrations over Time
Maternal plasma cortisol concentrations (ng/ml ± SEM) were measured from the beginning of the infusion period every 30 min for 2 hours and at 6 and 24 hours on gestational days 6, 40, 90 and 132. Cortisol concentrations in sheep within one treatment group were not different when compared between gestational days (not presented). Therefore, gestational day values were combined and treatment group responses over time are presented here. 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 (*).

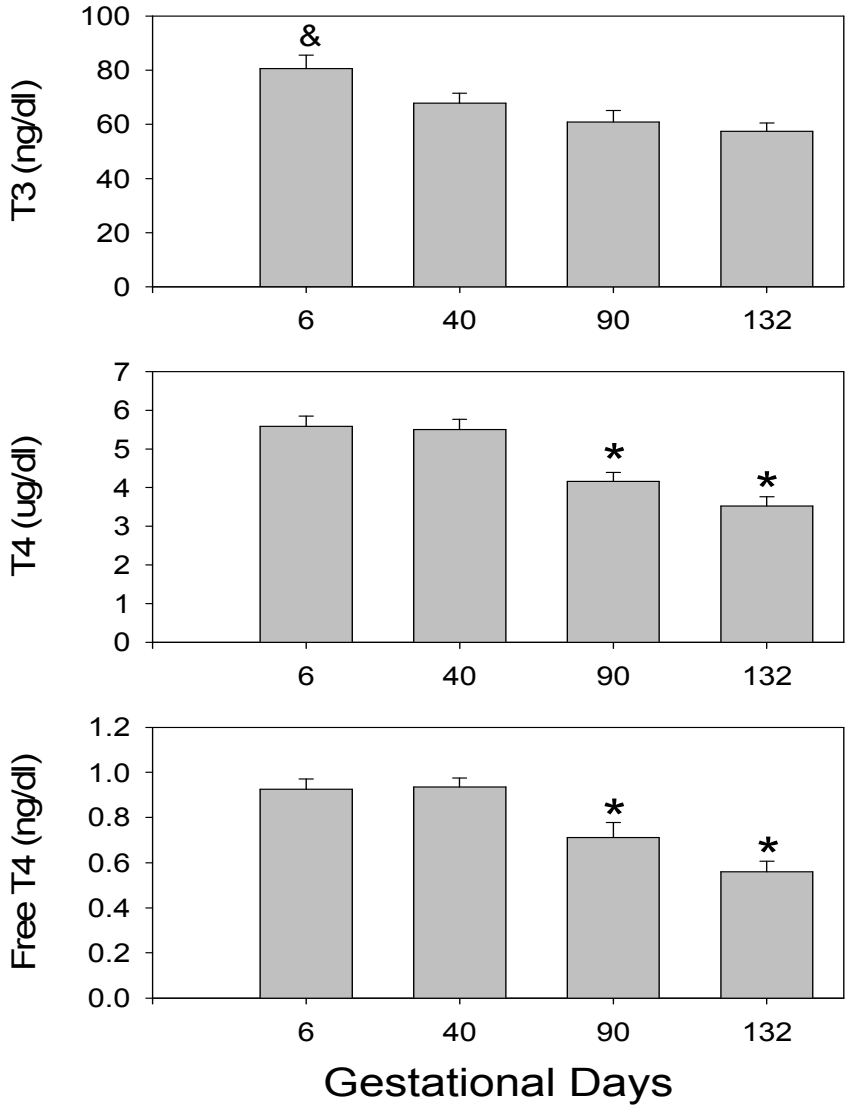

Figure 8: Maternal Plasma T_3, T_4 and free T_4 Concentrations on Different Gestational Days

This figure shows maternal plasma T_3 (ng/dl $\pm$ SEM), T_4 (μg/ml $\pm$ SEM) and free T_4 concentrations (ng/dl $\pm$ SEM) responses to infusions of saline or 0.75, 1.25 and 1.75 g/kg of alcohol on gestational days 6, 40, 90 and 132. There were no differences between treatment groups (not presented). Therefore, treatment group values were combined and gestational day responses are presented here. T3 was higher on gestational day 6 compared to all other gestational days (&). Total T4 and free T4 were lower on gestational days 90 and 132 compared to gestational days 6 and 40 (*).

CHAPTER IV

DISCUSSION

Alcohol stimulated the release of ACTH and cortisol in maternal sheep in a dose dependent fashion, with no effect of gestational day. Peak values for plasma ACTH and cortisol were detected at 2 hours after the beginning of the infusions. Plasma ACTH and cortisol returned to basal levels at 6 hours after the beginning of the infusions. Plasma ACTH and cortisol concentrations increased significantly in response to the infusion of the highest alcohol dose of 1.75 g/kg which resulted in peak BACs of 183 ± 5 mg/dl. This BAC can easily be achieved by abusers of alcohol. The results of this study are consistent with a number of studies investigating the HPA-axis response to alcohol in experimental animals (Zhang et al., 2005; Iqbal et al., 2005; Rivier, 1996). It has been shown that in adult rats alcohol exposure increases plasma ACTH and cortisol concentrations by stimulating the release of corticotropin releasing factor (CRF) from the hypothalamus (Rivier, 1996). There might also be a weak direct action of alcohol on the corticotrophs of the pituitary to release ACTH, but no evidence exists for alcohol actions on the adrenal cortex (Rivier, 1996).

We have previously reported that maternal sheep become hypoxemic, acidemic, and hypercapnic in response to the infusion of alcohol at a dose of 1.75 g/kg but not in response to lower doses (Cudd et al., 2001; Ramadoss et al., 2007). Hypoxemia stimulates the HPA-axis and acidemia and hypercapnea have been shown to increase plasma concentrations of glucocorticoids in adult animals (Augustinsson and Johansson, 1986; Perez, 1979). Therefore, in addition to the direct effect of alcohol on the

hypothalamus, there is also an indirect effect of alcohol on the HPA-axis, which might have augmented the ACTH and cortisol responses to the highest dose of alcohol. Characteristically, ACTH responds quickly to certain stimuli, such as psychogenic stress, novelty, hypoxia, or hypotension, for example. The release of cortisol follows, with a short delay of 15-30 minutes. Efforts were made in this study to avoid unduly stressing the sheep. As sheep are herd and prey animals and can easily feel threatened and stressed by being isolated from herdmates or by any noise or person approaching them. For the entire experimental period the sheep were kept inside the research facility in individual pens under controlled conditions, such as a regular 12 hr: 12 hr light/ dark cycle and regular feeding times. Sheep were within sight of their herdmates at any time. If ACTH had been released in response to stress, it would likely have caused a higher degree of variability in the observed plasma ACTH and cortisol concentrations both between and within subjects. The small variability observed suggests that the conditions under which the sheep were maintained provided a minimally stressful environment. Also novelty did not seem to have a great impact on ACTH and cortisol responses. There was no decline in plasma ACTH and cortisol concentrations with repeated treatments. This also shows that there was no development of tolerance of the HPA-axis response to alcohol and that with each maternal alcohol exposure the fetus was exposed to elevated circulating maternal cortisol concentrations. We previously reported that alcohol exposure during the human third trimester equivalent in sheep stimulates the maternal and fetal HPA-axis response and that maternal responses mirror those of the fetus (Cudd et al. 2001).

The current study extends our knowledge about the response of the maternal HPA-axis to alcohol exposure indicating that the HPA-axis remains activated with repeated alcohol exposure, throughout all three trimester equivalents.

The placenta presents a mechanical and biochemical barrier to many maternal factors that might be damaging to the fetus. However, alcohol can freely cross and enter the fetal circulation and thereby directly affect fetal tissues (Zhang et al., 2005). Small lipophilic molecules such as steroids (cortisol) and thyroid hormones also cross the placenta, but there are mechanisms in place that regulate the availability of these hormones. The placental enzyme 11-ß-hydroxy-steroid-dehydrogenase 2 (11-ß-HSD2) converts cortisol into its inactive form cortisone, protecting the fetus from elevated maternal plasma cortisol concentrations. In humans, guinea pigs, rats and sheep, activity of the ovine placental 11-ß-HSD2 decreases as gestation progresses (Clarke et al., 2002). Thus, especially late in gestation, during the time of brain and body growth spurt, the fetus might be exposed to increased maternally derived cortisol each time the mother consumes alcohol. An alcohol induced reduction of enzyme activity would presumably lead to increased transfer of maternal cortisol to the fetus. Exposure to high levels of cortisol during gestation has been associated with intrauterine growth retardation in experimental animals, non-human primates, and humans (Reinisch et al., 1978; Ikegami et al., 1997; French at al., 1999). There were no effects of treatment group on the weight of any fetal organ. However, there seemed to be a trend towards increased weights of the fetal liver in the higher alcohol dose groups (1.25 and 1.75 g/kg). Although this difference was not significant, it suggests that alcohol exposure may have impacted the

fetal liver. Liver damage is a known consequence of chronic alcohol consumption in humans and experimental animals and the degree of damage seems to be associated with the amount and duration of alcohol consumption (Walsh and Alexander, 2000). It is possible that at higher BACs than those achieved in our study, reductions of certain fetal tissues would occur.

There was no effect of alcohol on the release of thyroid hormones. Plasma concentrations of the thyroid hormones T_3, T_4, and free T_4 changed during pregnancy as a function of gestational day. Plasma concentrations of total T_4 and free T_4 were higher on gestational days 6 and 40 compared to gestational days 90 and 132. Plasma concentration of T_3 was highest on gestational day 6. Previous studies in pregnant women have shown that thyroid hormones change substantially during normal pregnancy. In normal women, the increased demands on the thyroid gland during pregnancy do not represent much of a load, but can lead to precipitation of clinical disease in females with subclinical hypothyroidism (Larsen et al., 1998).

During the first trimester the mother is the only source of thyroid hormones for the fetus, enabling the occupation of nuclear receptors for T_3 already present in the fetal cerebral cortex (de Escobar et al., 2004b). The plasma concentration of thyroxine binding globulin (TBG) increases in the first trimester. TBG is the major binding protein for T_4 and T_3, and serves to maintain a large circulating reservoir of these hormones. The change in the amount of TBG leads to a rise of total and free serum T_4 and T_3 levels, to about twice as high compared to the non-pregnant state. Free T_4 and T_3 return to normal levels at about midgestation and remain so until term. Since only T_4 is effectively taken

up by immature fetal brain tissue and converted to T_3 by two deiodinases, insufficient amounts of maternal free T_4 during this time would lead to a lack of T_3 in the fetal brain even if circulating maternal T_3 concentrations are normal (de Escobar et al., 2004b; Genuth, 1998b). The changes in T_4 and T_3 concentrations do not seem to depend on the hypothalamic-pituitary axis (HP-axis), as thyroid-stimulating hormone (TSH) from the hypothalamus is slightly decreased, and not increased as would be expected if the HP-axis were involved (Glinoer, 1990; Burrow, 1990).

The plasma concentrations of total and free T_4 observed in this study early in gestation, on GD 6 and 40, were higher than concentrations for total and free T_4 later in gestation, on GD 90 and 132. The plasma concentration of T_3 was highest on GD 6. This elevation of thyroid hormones observed early in pregnancy is most likely attributable to the physiological changes that take place during pregnancy, and not an alcohol effect, especially since there was no difference in thyroid hormone concentrations between sheep in the different treatment groups and the saline control group. One might have expected even higher values for T_3 and T_4 early in gestation if the same changes seen in pregnant women take place in the pregnant ewe. So far, no data are available regarding the physiological changes in thyroid gland function in sheep during gestation, and a comparison to reference values is not possible at this time. It has been reported that the most consistent effect of alcohol on the thyroid gland is a modest decrease in T_4 levels and a marked decrease in T_3 levels (Cicero, 1981).

There is evidence that the very low concentration of T_3 levels is not the result of altered thyroid gland function in response to alcohol, but rather due to a defect in the

peripheral conversion of T_4 to T_3, catalyzed by deiodinases (Cicero, 1981). Since alcohol can freely pass the placenta and reach the fetal circulation, it is possible that maternal alcohol exposure affects fetal deiodinases, which convert maternally derived T_4 into biologically active T_3. Impaired enzymatic activity could lead to a lack of fetal T_3, although maternal T_4 transfer is sufficient. Therefore, in order to properly assess maternal and fetal thyroid hormone responses to alcohol exposure, both maternal and fetal thyroid hormones need to be evaluated.

It is technically difficult to successfully instrument the ovine fetus on GD 42, the day on which we performed surgery, and to maintain the functionality of the vascular access until term, therefore only maternal values were evaluated in this study. In a different study performed in our laboratory, investigating maternal and fetal thyroid hormone responses to alcohol exposure during the third trimester equivalent, we identified reduced maternal T_3 concentrations on GD 132 in response to 1.75 g/kg alcohol, but no effect of alcohol on maternal T_4 or free T_4. Fetal T_3 levels in this study were elevated on GD 132.

There was no change in fetal free T_4, and total fetal T_4 was decreased early in the third trimester (GD 118), but not later (GD 132). The decreased level of maternal T_3 concentration in response to the highest alcohol dose suggests that peripheral conversion of T_4 to T_3 is impaired as a result of alcohol effects on the enzyme deiodinase. However, we did not find decreased T_3 levels in the fetus, indicating a defect in the conversion of T_4 to T_3. To clarify whether alcohol impairs the conversion of T_4 to T_3, the effects of alcohol on the enzyme catalyzing these reactions need to be investigated. There are several different isoforms of the enzyme deiodinase which might respond in slightly different ways to alcohol exposure and should be investigated individually to arrive at a final conclusion.

CHAPTER V

SUMMARY AND CONCLUSION

Pregnant sheep were exposed to alcohol throughout all three trimester equivalents using a binge drinking paradigm. The sheep were chronically instrumented to allow for intravenous infusion of the alcohol solutions and collection of blood samples for BAC and hormone analysis. Sheep were randomly assigned to receive one of three different alcohol solutions 0.75, 1.25, and 1.75 g/kg or physiological NaCl solution (0.9% NaCl). The respective infusions were administered on three consecutive days per week, followed by four days of no treatment, beginning on gestational day 4 and ending on gestational day 132. The respective infusions were given continuously over a one hour period of time. Blood samples were collected on gestational days 6, 40, 90, and 132 immediately before commencement of the infusion and every 30 minutes for the first two hours and then again 6 and 24 hours after the beginning of treatment. Sheep were euthanized and the fetuses collected for investigation on gestational day 133. The blood BAC values peaked at the end of the 1 hour infusion period. BAC values on the different gestational days were combined for ewes within one alcohol group as there was no difference. Sheep in the 0.75, 1.25 and 1.75 g/kg alcohol groups achieved BAC values of 93 ± 5, 126 ± 5 and 183 ± 5 respectively. Plasma concentrations of ACTH, cortisol, total T_4, free T_4, and T_3 were determined using specific radioimmunoassays.

Alcohol exposure lead to an increase of plasma ACTH and cortisol concentrations peaking at 2 hours after beginning of the infusion and returning to baseline values at 6 hours after beginning of the infusion. Plasma ACTH and cortisol

responses were not different on the different gestational days but differed between sheep of the different treatment groups. The 1.75 g/kg alcohol dose resulted in increased ACTH plasma concentrations at 1 and 1.5 hours after the beginning of the infusion compared to all other treatment groups except the 1.25 g/kg alcohol group. At 2 hours after the beginning of the infusions ACTH plasma concentrations in the 1.75 g/kg alcohol group were higher than in all other treatment groups.

Plasma cortisol concentrations of sheep receiving 1.75 g/kg alcohol were significantly elevated at 1, 1.5 and 2 hours after the beginning of the infusions compared to sheep of all other treatment groups

There was no difference in any of the plasma thyroid hormone concentrations between sheep in the different treatment groups. Thyroid hormone concentrations of sheep within one treatment group were different on the different gestational days. Plasma concentrations of total T_4 and free T_4 were higher on gestational days 6 and 40 compared to GDs 90 and 132, and plasma T_3 concentrations were highest on GD 6.

Based on the results of this study we conclude that alcohol stimulates the HPA-axis in a dose dependent fashion in pregnant sheep. There seems to be a minimum BAC necessary to evoke the HPA-axis response. The HPA-axis response remains unchanged with repeated alcohol exposure throughout pregnancy. Alcohol exposure did not seem to affect the release of thyroid hormones. Thyroid hormone concentrations changed during pregnancy unrelated to alcohol treatment.

REFERENCES

Abel, E.L., and Sokol, R.J. (1991). A revised conservative estimate of the incidence of FAS and its economic impact. *Alcohol Clin. Exp. Res. 15*, 514-524.

Augustinsson, O., and Johansson, K. (1986). Ammonium chloride induced acidosis and aldosterone secretion in the goat. *Acta. Physiol. Scand. 128*, 535-540.

Baumgartner, A., Rommelspacher, H., Otto, M., Schmidt, L.G., Kurten, I., Graf, K.-J., Campos-Barros, A., and Platz, W. (1994). Hypothalamic-pituitary-thyroid (HPT) axis in chronic alcoholism: I. HPT axis in chronic alcoholics during withdrawal and after 3 weeks of abstinence. *Alcohol Clin. Exp. Res. 18*, 284–294.

Bell, M.E., Wood, C.E., and Keller-Wood, M. (1991). Influence of reproductive state on pituitary-adrenal activity in the ewe. *Dom. Anim. Endocrin. 8*, 245-254.

Bloss, G., (1994). The economic cost of FAS. *Alcohol Health & Research World*, *18*, 53-54.

Burrow, G.N., (1990) Thyroid status in normal pregnancy. *J. Clin. Endocrinol. Metab. 71*, 274-275

Caetano, R, Ramisetty-Mikler, S., Floyd, L.R. and McGrath, C. (2006) The epidemiology of drinking among women of Child-Bearing Age. *Alcohol Clin. Exp. Res. 30*, 1023-1030.

Centers for Disease Control and Prevention (CDC). (2004) Fetal Alcohol Syndrome: Guidelines for referral and diagnosis.

Challis, J.R.G., Sloboda, D., Matthews, S.G., Holloway, A., Alfaidy, N., Patel, F.A., Whittle, W., Fraser, M., Moss, T.J.M., and Newnham, J. (2001). The fetal placental hypothalamic–pituitary–adrenal (HPA) axis, parturition and post natal health. *Mol. Cell. Endocrinol. 185*, 135–144.

Cicero, T.J. (1981). Neuroendocrinological effects of alcohol. *Annu. Rev. Med. 32*, 132-142.

Clarke, K.A., Ward, J.W., Forhead, A.J., Giussani, D.A., and Fowden, A.L. (2002). Regulation of 11β-hydroxysteroid dehydrogenase type 2 activity in ovine placenta by fetal cortisol. *J. Endocrinol. 172*, 527–534.

Cudd,T. A.; Chen,W.-J. A.; West, J. R. (2001). Fetal and maternal sheep hypothalamus pituitary adrenal axis responses to chronic binge ethanol exposure during the third trimester equivalent. *Alcohol Clin. Exp. Res. 25,* 1065-1071.

Cudd,T. A.; Chen,W.-J. A.; West, J. R. (2002). Fetal and maternal thyroid hormone responses to ethanol exposure during the third trimester equivalent of gestation in sheep. *Alcohol Clin. Exp. Res.26,* 53-58.

Cudd, T.A. (2005). Animal model systems for the study of alcohol teratology. *Exp. Biol. Med. 230*, 389 –393.

de Escobar, G.M., Obregon, M.J., and Rey, F.E. (2004a). Role of thyroid hormone during early brain development. *Europ. J. Endocrinol. 151*, U25-U37.

de Escobar, G.M., Obregon, M.J., and Rey, F.E. (2004b). Maternal thyroid hormones early in pregnancy and fetal brain development. *Best Pract.& Res. Clin. Endocrinol. 18*, 225-248.

de Kloet, E.R., Vreugdenhil, E., Oitzl, M.S., and Joels, M. (1998). Brain corticosteroid receptor balance in health and disease. *Endo. Rev. 19*, 269-301.

Delange, F.M. (1996) Endemic cretinism. In *The thyroid*, L.E. Braverman, R.D. Utiger, eds, (New York: J.B. Lippincott), pp. 756–765.

DeLong, G.R. (1996) The neuromuscular system and brain in hypothyroidism. In *The thyroid*, L.E. Braverman, R.D. Utiger, eds. (New York: J.B. Lippincott), pp. 826–835.

Fowden, A.L., and Forhead, A.J. (2004). Endocrine mechanisms of intrauterine programming. *Reproduction 127*, 515-526.

French, N.P., Hagan, R., Evans, S.F., Godfrey, M., and Newnham, J.P. (1999) Repeated antenatal corticosteroids: size at birth and subsequent development. *Am. J. Obstet. Gynecol. 180*, 114-121.

Genuth, S.M. (1998a). The hypothalamus and pituitary gland. In *Physiology* 5th edition, R.M. Berne, M.N. Levy, B.M., Koeppen, B.A. Stanton, eds, (Philadelphia, Elsevier), pp 819-859.

Genuth, S.M. (1998b). The Adrenal Glands. In *Physiology* 5th edition, R.M. Berne, M.N. Levy, B.M., Koeppen, B.A. Stanton, eds, (Philadelphia, Elsevier), pp 883-919.

Gubba, E.M., Netherton, C.M., and Herbert, J. (2000). Endangerment of the brain by glucocorticoids: Experimental and clinical evidence. *J. Neurocytol. 29*, 439-449.

Hennessy, D.P., Coghlan, J.P., Hardy, K.J., Scoggins, B.A., and Wintour, E.M. (1982). The origin of cortisol in the blood of fetal sheep. *J. Endocr. 95*, 71-79.

Hermann, D., Heinz, A., and Mann, K. (2002). Dysregulation of the hypothalamic–pituitary–thyroid axis in alcoholism. *Addiction 97*, 1369–1381.

Huang, W.L., Beazley, L.D., Quinlivan, J.A., Evans, S.F., Newnham, J.P., and Dunlop, S.A. (1999). Effect of corticosteroids on brain growth in fetal sheep. *Obstet. Gynecol. 94*, 213-218.

Huszenicza, G., Kulcsar, M., and Rudas, P. (2002). Clinical endocrinology of thyroid gland function in ruminants. *Vet. Med. – Czech. 47*, 199–210.

Ikegami, M., Jobe, A.H., Newnham, J., Polk, D.H., Willet, K.E., and Sly, P. (1997). Repetitive prenatal glucocorticoids improve lung function and decrease growth in preterm lambs. *Am. J. Resp. Crit. Care Med. 156*, 178-184.

Iqbal, U., Brien, J.F., Banjanin, S., Andrews, M.H., Matthews, S.G., and Reynolds, J.N. (2005). Chronic prenatal ethanol exposure alters glucocorticoid signalling in the hippocampus of the postnatal guinea pig. *J. Neuroendocrinol. 17*, 600-608.

Jones, K. L., Smith, D.W., Ullbiand, C.N., and Streissguth, A.P. (1973). Pattern of malformation in offspring of chronic alcoholic mothers. *Lancet I*, 1267-1271.

Keller-Wood, M., Cudd, T.A., Norman, W., Caldwell, S.M., and Wood C.E. (1998). Sheep model for study of maternal adrenal gland function during pregnancy. *Lab. Anim. Sci. 48*, 507-512.

Kim, C. K., Turnbull, A. V., Lee, S. Y., and Rivier, C. L. (1999). Effects of prenatal exposure to alcohol on the release of adenocorticotropic hormone, corticosterone, and proinflammatory cytokines. *Alcohol Clin. Exp. Res.23,* 52-59.

Koibuchi, N., and Chin, W.W. (2000). Thyroid hormone action and brain development. *Trends Endocrinol. Metab. 11*, 123–128.

Kornguth, S.E., Rutledge, J.J., Sunderland, E., Siegel, F., Carlson, I., Smollens, J., Juhl, U., and Young, B. (1979). Impeded cerebellar development and reduced serum thyroxine levels associated with fetal alcohol intoxication. *Brain Res. 177*, 347-360.

Larsen, P.R., Davies, T.F., and Hay, I.D. (1998) The thyroid gland. In *Williams Textbook of Endocrinology*, 9th edition, J.D. Wilson, D.W. Foster H.M. Kronenberg and P.R. Larsen, eds. (WB Saunders, Philadelphia), pp 389-515.

Lemoine, P., Harousseau, H ., Borteyru,J.-P., and Menuet, J.-C. (1968). Les enfants de parents alcoholiques. Anomalies observees [Children of alcoholic patients. Observed Anomalies]. *Ouest.- Medical*. *21*, 476-182.

Liggins, G.C. (1974). Parturition in the sheep and the human. In *Physiology and Genetics of Reproduction*. E.M. Coutinho and F. Fuchs, eds. (Plenum Press, New York).

Liggins, G.C. (1994). The role of cortisol in preparing the fetus for birth. *Reprod. Fertil. Dev. 6*, 141-150.

Man, E.B., Serunian, S.A. (1976). Thyroid function in human pregnancy. *Am. J. Obstet. Gynecol. 125*, 949-957.

Matthews, S.G. (2000). Antenatal glucocorticoids and programming of the developing CNS. *Pediatric Research. 47*, 291-300.

Mukamal, K.J., Conigrave, K.M., Mittleman, M.A., Camargo, C.A., Stampfer, M.J. Willett, W.C. and Rimm, E.B. (2003). Roles of drinking pattern and type of alcohol consumed in coronary heart disease in men. *N. Engl. J. Med. 348*, 109-18.

National Institute on Alcohol Abuse and Alcoholism (NIAAA). (2000) *Alcohol Alert*, No. 50.

Nolten,W. E., and P. A. Ruekert. (1981). Elevated free cortisol index in pregnancy: possible regulatory mechanisms. *Am. J. Obstet. Gynecol. 139*, 492–498.

Ogilvie, K., Lee, S., and Rivier, C. (1997). Effect of three different modes of alcohol administration on the activity of the rat hypothalamic-pituitary-adrenal axis. *Alcohol Clin. Exp. Res. 20*, 467-476.

Oppenheimer, J.H., and Schwartz, H.L. (1997). Molecular basis of thyroid hormone-` dependent brain development. *Endocr. Rev. 18*, 462-75.

Perez, G.O., Kem, D.C., Oster, J.R., and Vaamaonde, C.A. (1979). The effect of acute metabolic acidosis on plasma cortisol, rennin activity, and aldosterone. *Hormone Res. 11*, 12-21.

Portolés, M., Sanchis, R., and Guerri, C. (1985). Effect of chronic and acute ethanol ethanol administration on plasma thyroid hormone levels in the rat. *IRCS Med. Sci. 13*, 615-616.

Ramadoss, J., Lunde, E.R., Pina, K.B., Chen, W.-J., and Cudd, T.A. (2007). All three trimester binge alcohol exposure causes fetal cerebellar purkinje cell loss in the presence of maternal hypercapnea, acidemia, and normoxemia: Ovine model. *Alcohol Clin. Exp. Res*. *31***,** 1252–1258.

Reinisch, J.M., Simon, N.G., Karow, W.G., and Gandelman, R. (1978) Prenatal exposure to prednisone in humans and animals retards intrauterine growth. *Science 202*, 436-438.

Riley, P.R., and McGee, C.L. (2005). Fetal alcohol spectrum disorders: An overview with emphasis on changes in brain and behavior. *Exp. Biol. Med. 230*, 357–365.

Rivier, C. (1996). Alcohol stimulates ACTH secretion in the rat: Mechanisms of action and interactions with other stimuli. *Alcohol Clin. Exp. Res*. *20***,** 240–524.

Ruiz, U., Piasecki, G.J., Balogh, K., Polansky, B.J., and Jackson, B.T. (1972). An experimental model for fetal pulmonary hypertension. A preliminary report. *Am. J. Surg. 123*, 468-471.

Seckl, J.R. (2004). Prenatal glucocorticoids and long-term programming. *Europ. J. Endocrinol. 151*, U49-U62

Streissguth, A.P., and Little, R.E. (1994). Alcohol, pregnancy, and the Fetal Alcohol Syndrome. In *Biomedical Education: Alcohol Use and Its Medical Consequences.* 2nd Edition. Unit 5 of Alcohol use and its medical consequences. A comprehensive slide teaching program for biomedical education. Dartmouth Medical School.

Tabakoff, B., and Hoffman, P.L. (2000). Animal models in alcohol research. *Alcohol Res. Health 24*, 77–84.

Walsh, K., and Alexander, G. (2000) Alcoholic liver disease. *Postgrad. Med. J. 76*, 280-286.

Weinberg, J. (1989). Prenatal ethanol exposure alters adrenocortical development of offspring. *Alcohol Clin. Exp. Res*. *13*, 73:83.

Wood, C.E., and Chen, H.-G. (1989). Acidemia stimulates ACTH, vasopressin, and heart rate responses in fetal sheep. *Am. J. Physiol. Regul. Integr. Comp. Physiol. 257*, R344-R349.

Zhang, X., Sliwowska, J.H., and Weinberg, J. (2005). Prenatal alcohol exposure and fetal programming: Effects on neuroendocrine and immune function. *Experimental Exp. Biol. Med.* 376-388.

Zoeller, R.T., Dowling, A.L.S., Herzig, C.T.A., Iannacone, E.A., Gauger, K.J., and Bansal, R. (2002). Thyroid hormone, brain development, and the environment.*Env.Health Persp. 110*, 355-365.

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