Published in final edited form as:
Alcohol. 2013 February ; 47(1): . doi:10.1016/j.alcohol.2012.10.004.

THE ROLE OF CORTISOL IN CHRONIC BINGE ALCOHOL-INDUCED CEREBELLAR INJURY: OVINE MODEL

Shannon E. Washburn[1], **Ursula Tress**[2], **Emilie R. Lunde**[1], **Wei-Jung A. Chen**[3], and **Timothy A. Cudd**[1]

[1]Department of Veterinary Physiology and Pharmacology and Michael E. DeBakey Institute, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas, 77843, USA

[2]Laboklin GmbH, Veterinary Laboratory Diagnostic, Steubenstr. 7, 97688 Bad Kissingen, Germany

[3]Department of Neuroscience and Experimental Therapeutics, Texas A&M Health Science Center, Bryan, Texas, 77807, USA

Abstract

Women who drink alcohol during pregnancy are at high risk of giving birth to children with neurodevelopmental disorders. Previous reports from our laboratory have shown that third trimester equivalent binge alcohol exposure at a dose of 1.75 g/kg/day results in significant fetal cerebellar Purkinje cell loss in fetal sheep and that both maternal and fetal adrenocorticotropin (ACTH) and cortisol levels are elevated in response to alcohol treatment. In this study, we hypothesized that repeated elevations in cortisol from chronic binge alcohol are responsible at least in part for fetal neuronal deficits. Animals were divided into four treatment groups: normal control, pair-fed saline control, alcohol and cortisol. The magnitude of elevation in cortisol in response to alcohol was mimicked in the cortisol group by infusing pregnant ewes with hydrocortisone for 6 hours on each day of the experiment, and administering saline during the first hour in lieu of alcohol. The experiment was conducted on three consecutive days followed by four days without treatment beginning on gestational day (GD) 109 until GD 132. Peak maternal blood alcohol concentration in the alcohol group was 239 ± 7 mg/dl. The fetal brains were collected and processed for stereological cell counting on GD 133. The estimated total number of fetal cerebellar Purkinje cells, the reference volume and the Purkinje cell density were not altered in response to glucocorticoid infusion in the absence of alcohol. These results suggest that glucocorticoids independently during the third trimester equivalent may not produce fetal cerebellar Purkinje cell loss. However, the elevations in cortisol along with other changes induced by alcohol could together lead to brain injury seen in the fetal alcohol spectrum disorders.

Keywords

Fetal Alcohol Syndrome; ethanol; sheep; cortisol; cerebellum; Fetal Alcohol Spectrum Disorder

Send correspondence to: Shannon E. Washburn, DVM, PhD, Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University 4466, College Station, TX 77843-4466, Phone: 979-845-1993, Fax : 979-845-6544, swashburn@cvm.tamu.edu.

Introduction

The first scientific report linking maternal consumption of alcohol during pregnancy with harmful abnormalities and malformations in the child was in 1968 (Lemoine et al., 2003). These abnormalities and malformations were further characterized and refined by the Pediatric Dysmorphology Unit at the University Of Washington School Of Medicine a few years later, and the term Fetal Alcohol Syndrome (FAS) was used to describe affected children (Jones and Smith, 1973; Jones et al., 1974; Jones et al., 1973). Standardized diagnostic criteria for FAS were later adopted with the recommendation that FAS be diagnosed when the patient had signs in the three following categories: growth retardation, central nervous system abnormalities, and characteristic facial dysmorphology (Clarren and Smith, 1978; Rosett, 1980). It was soon realized that there were affected individuals that had structural anomalies and behavioral and neurocognitive disabilities that did not meet this diagnostic criteria, so the term alcohol related birth defects (ARBD) evolved (Sokol and Clarren, 1989), followed by a refinement of diagnostic criteria by the Institute of Medicine (IOM) in 1996 and use of the term alcohol related neurodevelopment disorder ARND (Institute, 1996). Finally, the IOM criteria were once again further clarified by use of the all-inclusive umbrella term Fetal Alcohol Spectrum Disorder (FASD) which describes all of the adverse effects of alcohol on the developing child on a spectrum, with FAS being the most severe end of the spectrum (Hoyme et al., 2005; Sokol et al., 2003).

The terminology and classification is important, because when considering all ranges of damage in FASD, a more accurate assessment of the prevalence of individuals affected by prenatal alcohol exposure can be made. Estimating the prevalence of FAS alone (the most severe end of the spectrum in FASD) underestimates the impact of this problem. Prevalence rates for FAS have been reported as 0.5–2.0 per 1000 live births with prevalence rates for FASD estimated as high as 10 per 1000 live births (May and Gossage, 2001). The cost of FAS in the United States has been estimated at more than $4 billion annually, or $2.9 million per individual (Lupton et al., 2004).

With documentation of the link between maternal consumption of alcohol during pregnancy and harmful effects to the unborn child, physicians were advised to counsel pregnant women to reduce the risk of harm to their child by abstaining from alcohol during their pregnancy (Rosett, 1980). In the years that have followed, considerable effort has been made to educate women about the dangers of drinking during pregnancy, however drinking rates among women of child bearing age and the prevalence rate of FAS have changed little (Caetano et al., 2006). Since the magnitude, impact and cost of FASD is great and educational efforts have failed to reduce the incidence, it is necessary to explore the development of intervention or amelioration strategies and therapeutics. In order to accomplish this, the specific mechanisms by which alcohol mediates neurodevelopmental damage must be identified.

Investigation of the effects of maternal alcohol consumption on the adrenocortical hormones began not long after the documentation of FAS (Kakihana et al., 1980). Alcohol can interfere with fetal development directly by crossing the placenta and entering fetal circulation and/or by disturbing maternal endocrine function and maternal-fetal hormone interaction (Gabriel et al., 1998). Prenatal alcohol exposure has been demonstrated to result in elevated cortisol levels in human infants (Jacobson et al., 1999) and in animal models including neonatal rats (Weinberg, 1989), primates (Schneider et al., 2002), and fetal sheep (Cudd et al., 2001a). While normal cortisol concentrations are vital for normal brain development and somatic growth, increases in cortisol during development can interfere with neuronal growth, proliferation and differentiation, expression of neuronal and glial antigens and myelination (Bohn, 1984).

Glucocorticoid administration to women in preterm labor, or directly to preterm infants, has been widely used between the 24th and 34th week of gestation to help reduce the incidence of respiratory distress in preterm infants (NIH, 1995). The National Institute of Health Consensus Conference recommends a single administration of antenatal corticosteroids to women at risk for preterm birth, but advises against routine repeat courses of corticosteroids due to insufficient data regarding efficacy and safety (Gilstrap et al., 2001). Growing evidence indicates that the administration of glucocorticoids during the perinatal period can result in long-term damage, including altered brain architecture (McGowan et al., 2000; Murphy et al., 2001) and behavioral and cognitive deficits (Jameson et al., 2006). High levels of glucocorticoids have been shown to cause an alteration in hippocampal development and learning ability (Bodnoff et al., 1995). In sheep, repeated prenatal corticosteroid administration has demonstrated reduction in brain weights, disruption of optic nerve and corpus callosum myelination, and delayed astrocyte and capillary tight junction maturation (Huang et al., 1999; Huang et al., 2001a, b) and in an intrauterine growth restricted fetal sheep model, glucocorticoid administration worsened growth restriction and caused brain injury (Miller et al., 2007). Single dose glucocorticoid administration in rhesus monkeys has demonstrated significant dose dependent degeneration of CA1-CA3 hippocampal pyramidal neurons (Uno et al., 1994) and significantly reduced cortical folding and decreased surface area has been reported in infants exposed to repeated doses of antenatal glucocorticoids (Modi et al., 2001).

In addition to brain abnormalities, a single dose of antenatal corticosteroids has been shown to cause growth restriction in lambs (Jobe et al., 1998) and a decrease in protein accretion in the fetus (Milley, 1995). Multiple dose glucocorticoid administration has been shown to cause reduction of head circumference and birth weight in humans (Abbasi et al., 2000) and multiple dose administration of prenatal glucocorticoids at a dose that is three-fold lower than what women in preterm labor receive resulted in decreased fetal birth weight and decreased newborn brain weight and biparietal diameter in sheep (Kutzler et al., 2004).

Similarities in brain responses have been noted between fetal alcohol exposure and perinatal exposure to high glucocorticoid levels, suggesting that an alteration in maternal and/or fetal hypothalamus-pituitary-adrenal (HPA) axis activity and glucocorticoid levels may be responsible in part for the neurobehavioral deficits seen with prenatal alcohol exposure (Gabriel et al., 1998; Kim et al., 1999; Weinberg, 1989). The nature of the effect of the increase in maternal glucocorticoids depends on the developmental stage at which exposure occurs. In animals that give birth to relatively mature young, such as the sheep and primate, maximal brain growth and a large proportion of neuroendocrine maturation takes place in utero (Edwards and Burnham, 2001) thus making the sheep a good translational model choice for this experiment (Wilson and Cudd, 2011).

Purkinje cells are widely studied in neuronal investigations and are one of the most important cells in the cerebellum since they have many connections with different areas in the brain, are the only output neuron of the cerebellar cortex and are involved in many vital functions, including the coordination of sensory input and motor movement, memory, analysis of auditory information, and neurosteroid formation (Salouci et al., 2012). Although extensive research in the field of alcohol neuoroteratogenesis has demonstrated a detrimental effect on developing Purkinje cells (Cragg and Phillips, 1985; Goodlett and Eilers, 1997; Goodlett et al., 1990b; Goodlett et al., 1998; Light et al., 2002a; Maier et al., 1999; Maier and West, 2001; Pauli et al., 1995; Ramadoss et al., 2007a; Ramadoss et al., 2007b) , and the cerebellum appears to be particularly vulnerable to alcohol exposure during development (Autti-Ramo et al., 2002; Bonthius and West, 1990; Goodlett et al., 1990a; Goodlett et al., 1990b; Hamre and West, 1993; Light et al., 2002b; Ramadoss et al., 2007a; Ramadoss et al., 2007b; West et al., 1990), little is known about underlying potential mechanisms for these

effects. These factors, the ease with which Purkinje cells can be counted and the predictability of Purkinje neuron loss with prenatal alcohol exposure makes them a good marker for damage and a tool to study the mechanism by which alcohol mediates neurodevelopmental damage.

There is a degree of unpredictability when trying to anticipate alcohol-induced cell loss because prenatal alcohol exposure is known to have temporal and regional variation in the damage it causes and neurons in the brain are dissimilarly vulnerable to alcohol-induced cell loss (Maier et al., 1999; Maier and West, 2001). The aim of this study was not to characterize the effects of alcohol or corticosteroids on various brain regions, but rather to investigate the potential role of corticosteroids in the mechanism of Purkinje cell loss, a neuron known to be highly and consistently vulnerable to alcohol exposure with reliable, well established decreases in neuron number as a measurable effect (Bonthius and West, 1990; Goodlett et al., 1990b; Hamre and West, 1993; Maier et al., 1999; Maier and West, 2001; Ramadoss et al., 2007a; Ramadoss et al., 2008; Ramadoss et al., 2007b; West et al., 2001)

Previous reports from our laboratory have shown that third trimester equivalent binge alcohol exposure at a dose of 1.75 g/kg (which creates blood alcohol concentrations easily achievable by women who abuse alcohol during pregnancy) results in significant cerebellar Purkinje cell loss in fetal sheep (Ramadoss et al., 2007a; Ramadoss et al., 2007b; West et al., 2001) and also results in a significant increase in both maternal and fetal adrenocorticotropin (ACTH) and cortisol levels (Cudd et al., 2001b; Ramadoss et al., 2008). The fetus is exposed to increases in cortisol with every maternal alcohol binge episode, and these alcohol mediated increases in fetal cortisol may represent a mechanism of brain injury. In this study, we hypothesized that repeated elevations in cortisol from chronic binge alcohol exposure are responsible, at least in part, for fetal neuronal deficits and that creating the pattern of cortisol concentration increases in the absence of alcohol would lead to Purkinje cell loss.

Methods

Animals and Breeding

Pregnant ewes were divided in to 4 groups: normal control, saline control, alcohol and cortisol, with a final n= 7 sheep per group. The experimental procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M University. Suffolk ewes (aged 2 to 5 years with an average age of 3.2 years old) 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 20 mg of prostaglandin F2α (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 to confirm pregnancy on gestational day (GD) 25 and GD 90. Conditions of constant temperature (22°C) and fixed light dark cycle (12:12) were maintained. Subjects received 2 kg/day of a complete ration (Sheep and Goat Pellet, Producers Cooperative Association, Bryan, TX).

Alcohol and Cortisol Dosing Protocol

The alcohol group received an alcohol dosage of 1.75 g/kg body weight. Alcohol infusions were 40% w/v in sterile saline and administered intravenously over one hour. Infusions were administered on three consecutive days followed by four days without alcohol beginning on

day 109 of gestation and continuing until GD132 which resulted in a total of four binge cycles, or 12 infusions (Figure 1). This paradigm of administration mimics the binge drinking pattern commonly observed in women who consume alcohol when pregnant (Caetano et al., 2006; Ebrahim et al., 1999; Gladstone et al., 1996). The saline and cortisol group received 0.9% saline of a volume and at an infusion rate equivalent to that of the alcohol dose received by the alcohol group. Infusion solutions were delivered intravenously by peristaltic pump (Masterflex, model 7014-20, Cole Parmer, Niles IL) and pumps were calibrated before the infusion.

The magnitude of elevation in maternal cortisol in response to alcohol (Cudd et al., 2001b) was mimicked in the cortisol group by infusing hydrocortisone for 6 hours on each day of the experiment. Determination of the cortisol infusion rate was based on the metabolic clearance rate for cortisol in sheep (Wood, 1987), and empirical pilot data verifying that the resultant plasma cortisol values were like those resulting from the alcohol infusion paradigm. The infusion rate to create levels matching those seen in response to alcohol (Cudd et al., 2001b) was titrated and averaged 0.8 μg/kg/min for the first two hours, followed by 0.4 μg/kg/min for hours 3 through 6. Blood samples were drawn for measurement of plasma cortisol concentration at 0, 1 hour, 2 hours, and 6 hours (and in between time points as needed) and the infusion was titrated to match the cortisol concentrations present in the alcohol group. Solu-Cortef® (hydrocortisone sodium succinate for injection, USP, Pharmacia and Upjohn, Kalamazoo, MI) was diluted in 0.9% saline and administered via a syringe pump (Harvard Apparatus syringe pump Model 964).

Experiment Protocol

The alcohol or cortisol treatment regimens began on GD 109 and were terminated on GD 132. On GD 104, the ewes underwent surgery to implant maternal femoral arterial and venous poly-vinyl chloride catheters (0.05” inner diameter, 0.09” outer diameter) as previously described (Cudd et al., 2001a); these catheters remained in place for the duration of the experiment. In brief, anesthesia was induced by administering diazepam (0.2mg/kg; Abbott Laboratories, North Chicago, IL) and ketamine (4 mg/kg; Ketaset®; Fort Dodge, IA) intravenously. Intubation followed, and a surgical plane of anesthesia was maintained using isoflurane (0.5% to 2.5%; IsoFlo®; Abbott Laboratories) and oxygen. The 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 ewe received an injection of flunixin meglumine, a prostaglandin synthase inhibitor, intravenously to reduce postoperative pain (1.1 mg/kg; Banamine®; Scherring-Plough, Union, NJ). Ewes also received the postoperative antibiotics ampicillin trihydrate (25 mg/kg administered subcutaneously for 5 days, Polyflex®, Aveco, Fort Dodge, IA) and gentamicin sulfate (2 mg/kg administered intramuscularly twice daily for 5 days; Gentavet®, Velco, St. Louis, MO).

Efforts were made to avoid unduly stressing the sheep, thus they were within sight of their herdmates at all times, including during the experiment. On the days of experiment, the vascular catheter lines were run behind a curtain that prevented the sheep from seeing the experimenter. This set up allowed administration of infusions and sampling without the sheep being handled or observing the presence of the experimenter. On the days of infusions, ewes were connected to the infusion pump by 0830 hr and alcohol or saline was infused continuously over 1 hr, between 0830 and 0930 hr. In the cortisol group, the magnitude of the maternal elevation of cortisol in response to alcohol was mimicked by infusing hydrocortisone for 6 hours. Infusions were given through the venous catheter and blood was sampled from the arterial catheter. Subjects were sacrificed on GD 133.

Blood Alcohol Concentration (BAC) and Cortisol Measurement

Blood was drawn from the femoral artery catheter on GD 109, 116, 123 and 132, one hour following the commencement of alcohol infusions (the time at which BAC is known to peak) for the measurement of BAC. A 20 μl aliquot of blood was collected into a microcapillary tube and transferred into a vial 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 hours 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).

Blood samples (4 ml) for measurement of plasma cortisol concentration were collected into chilled polystyrene tubes containing ethylenediaminetetraacetic acid at the 0, 1, 2 and 6 hour time points. The tubes were kept in ice water until centrifugation (20 minutes at 2800 × g at 4°C). Plasma was pipetted off and stored in aliquots at −20°C. Plasma cortisol concentration was measured by specific radioimmunoassay using a commercial kit (Cortisol Coat-a-Count™, Siemens Healthcare Diagnostics, Los Angeles, California) as previously described (Cudd et al., 1996).

Fetal Cerebellar Tissue Processing

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

At the time of processing, the cerebellum was removed and the tissues were 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; HistoresinTM Embedding kit, Leica, Wetzlar, Germany). Following infiltration, the tissue was embedded in a solution containing 1 ml of 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 using a microtome (model RM2255, Leica, Nussloch, Germany). Sections were then mounted serially on glass slides, stained with cresyl violet, and coverslipped.

Stereological Cell Counting

The total number of Purkinje cells in the whole cerebellum was estimated using an unbiased stereological cell counting technique as described in previous publications (West et al., 2001). The Nikon (Garden City, NY) Optiphot microscope used in this study had a 4× objective lens for volume measures and a 60× 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 Cavalieri's Principle and was calculated by the equation $Vref = \Sigma pi \times A(pi) \times t$ where Σpi is the total number of points (pi) counted, A(pi) is the known area associated with each point, and t is the known distance between two serial sections counted. 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 $Nv = \Sigma Q / (\Sigma disector \times A(fr) \times h)$ 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 respective regions and the numerical density of cells within this reference volume as described before (West et al., 2001).

Statistical Analysis

Maternal plasma cortisol levels at the end of each week were analyzed using repeated measure analysis of variance (ANOVA), with the treatment group and week of exposure as independent grouping factors and the time as a within subject factor. A one-way ANOVA was performed on maternal cortisol levels at different time points within each treatment group and vice-versa. Further pairwise comparisons were done using Student-Newman-Keuls method. Data are presented as mean ± SEM. Fetal weight data and Purkinje cell number were subjected to a one way analysis of variance with treatment group as the sole independent variable. Significant main effects or interactions were subjected to multiple comparison testing with Fisher's least significant difference test. The alpha value for any statistical test was set at 0.05.

Results

The mean blood alcohol concentration in the alcohol group was 239 ± 7 mg/dl; there were no statistical differences across days of sampling.

Fetal weight and body length did not differ among groups ($p = 0.8$ and 0.89 respectively); the cortisol and alcohol group exhibited a lower mean adrenal weight than the saline or normal controls, but there was not a statistical differences between groups ($p = 0.08$) (Figure 2).

A repeated measure ANOVA on the maternal cortisol levels confirmed a significant main effect of time ($p<0.001$), treatment group ($p<0.001$) and a significant interaction between time and treatment group ($p<0.001$). No main or interactive effect of week of exposure was observed, which indicates that cortisol level in experiment animals were not significantly different between weeks. Since no significant effect of week of exposure was observed on maternal cortisol levels, values at all 4 weeks of exposures were combined for the each 4 time points and analyzed using one-way ANOVA between the time points and each treatment group.

The results of a one-way ANOVA on maternal cortisol levels at 0, 60, 120 and 360 minutes showed no significant difference between time points for the saline control group. For both the alcohol and cortisol group, maternal cortisol levels at different time points were statistically different ($p<0.001$) and further pairwise comparison indicated that the cortisol level at 120 minutes was significantly elevated compared to the 0, 60 and 360 minutes ($p<0.001$). Maternal cortisol levels at the other time points were not different between the alcohol and cortisol group, which implies that the elevation in maternal cortisol level due to alcohol infusion or hydrocortisone injection was transient and it returned to base line within 6 hours. The results of a one-way ANOVA on maternal cortisol levels at 0, 60, 120, and 360 minutes showed a significant difference between groups at 120 minutes for the alcohol and cortisol groups compared to the saline control ($p<0.001$).

In summary, maternal plasma cortisol concentrations on each experiment day were significantly increased in the alcohol and cortisol group compared to the saline control group

at 120 minutes but the alcohol and cortisol groups did not differ significantly from each other at any time point (Figure 3 shows a representative graph of cortisol levels from GD 132).

Fetal cerebellar Purkinje cell number (Figure 4) differed significantly among groups, with alcohol differing from saline and normal control groups ($p = 0.008$ and 0.038 respectively). The cortisol group did not differ from the alcohol group ($p = 0.06$) nor the saline or normal control groups ($p = 0.259$ and 0.601 respectively). There were no significant differences between the saline and normal control groups.

Discussion

This study provides important in vivo, highly translational information on the effect of chronic alcohol induced increases in maternal and fetal glucocorticoid levels during the brain growth spurt in order to further progress understanding of the mechanism by which alcohol mediates neurodevelopmental damage during this time period. We hypothesized that the repeated elevations in cortisol that occur with chronic binge alcohol exposure are responsible at least in part for the fetal Purkinje neuronal deficits that occur with prenatal alcohol exposure. The estimated total number of fetal cerebellar Purkinje cells, the reference volume and the Purkinje cell density were not altered in response to chronic glucocorticoid infusion in the absence of alcohol. The fetal body weight and length were not different among groups.

Neurodevelopmental damage to the fetus from prenatal alcohol exposure or from alcohol induced increases in glucocorticoids may involve the intrauterine environment and/or maternal interactions and thus the sheep model, where the entire gestational equivalent of human brain development and a large proportion of neuroendocrine maturation occurs in utero, is a highly translational model choice to address these questions (Edwards and Burnham, 2001; Wilson and Cudd, 2011). There would be less clinical correlation with the use of small laboratory mammals since the corticosteroid treatment would occupy a large portion of their short gestation and rapid postnatal development period (Huang et al., 1999) and because variations in the route of administration of glucocorticoids (maternal versus direct injection into the fetus or neonate) are now known to alter outcome (Huang et al., 1999; Newnham et al., 1999). The sheep model allows maternal administration thus again more closely paralleling human conditions. The unique dosing protocol of our study (6 hour infusion on 12 days across the third trimester equivalent) is a novel approach compared to the typical protocol employed in the majority of work examining the effects of exogenously administered glucocorticoids which usually consist of a series of one to three single injections.

These differences between the sheep and rodent model as well as the different developmental periods and the variations in corticosteroid dosing paradigms and stereologic neuron counting methods across studies must be kept in mind when comparing our results with work done in the rodent model. Rugerio and coworkers concluded that glucocorticoids alter the trajectory of Purkinje neuron development (as evidenced by visible electron microscopy changes in the Purkinje cells) soon after birth in rats exposed to single doses of corticosterone on GD 17–19 (Rugerio-Vargas et al., 2007). The brains were collected from the rats on postnatal day 12, which is comparable to the time of birth in humans and the same development end point used in our study. Similar to our results, they found no change in Purkinje cell number between treated and control groups, but their counts were performed only in Lobe II of the cerebellum (Rugerio-Vargas et al., 2007). Velasquez and Romano also reported a modifying effect on cerebellar development (a suggested accelerated rate of maturation) with administration of corticosterone to pregnant rats on GD17–19 though their

investigation was limited to changes observed in the granule cell layer on postnatal day 6 and 12 and Purkinje cells were not examined (Velazquez and Romano, 1987). Another study conducted in the mouse model found that 3 single doses of corticosteroid administered to neonatal mice resulted in long-term neuromotor deficits on complex wheel activity tasks and a decrease in granule neurons of the internal granule layer, but no change in Purkinje cell number, however brains were not collected for examination until 190 days of age (i.e., adults) (Maloney et al., 2011). Alcohol induced alteration of neurotrophic mediated processes with subsequent deficits in Purkinje cell structure and activity has also been documented in the rat (Gonzalez-Burgos and Alejandre-Gomez, 2005). These findings in the rodent model together with our results suggest that exposure to increased levels of glucocorticoids prenatally may alter cerebellar development, neurotrophic factors, and Purkinje cell morphology or function, but a decrease in Purkinje cell neuron numbers may not be observed.

Administration of cortisol maternally, as administered in this study, can reasonably be expected to increase fetal cortisol levels. The placenta presents a complete barrier to ACTH (Jones, et al., 1975) and a partial barrier to cortisol (Beitins et al., 1970) with estimates of approximately 10–20% of maternal cortisol crossing the placenta to the fetus in humans (Gitau et al., 1998; Murphy et al., 2006). In human pregnancy, endogenous maternal cortisol concentrations are 5–10 times higher than fetal cortisol concentrations. Placental 11 β-hydroxysteroid-dehydrogenase activity, an enzyme that converts cortisol to an inactive metabolite, maintains this maternal: fetal difference and protects the fetus from the high maternal concentrations of endogenous glucocorticoids (Murphy et al., 2006). However, since fetal concentrations of cortisol are much lower than maternal concentrations, a 10–20% elevation in maternal cortisol level could double fetal concentrations (Gitau et al., 1998; Murphy et al., 2006) and maternal cortisol concentrations have been reported as accounting for as much as 40% of the variance in cortisol concentration in the human fetus (Gitau et al., 1998). In addition, 11 β-hydroxysteroid-dehydrogenase has a low affinity for synthetic glucocorticoids, so they can pass rapidly from the mother to the fetus (Edwards and Burnham, 2001).

While concurrent measurement of both maternal and fetal cortisol levels would have been ideal in this study to confirm and more accurately compare the fetal exposure level, fetal surgery would have had to be performed during the course of the experimental period. The fetus cannot be instrumented before GD 113, which is during the third trimester equivalent and thus during the time of this experiment (GD 109–133). This would have introduced a potential confound on cortisol levels from surgery and instrumentation during the experiment. Maternal and fetal plasma cortisol levels have been shown to have a linear correlation (Gitau et al., 1998) and both maternal and fetal cortisol concentrations in response to alcohol have been characterized in the sheep model (Cudd et al., 2001b), so any changes observed from the cortisol treatment of the ewe may be attributed to direct effects on the fetus as predicted.

Utilizing the third trimester equivalent of human pregnancy in sheep for this experiment allowed assessment during a period of rapid fetal growth (and the brain growth spurt) before the increases fetal adrenal secretion of cortisol associated with parturition occur (Jensen et al., 2005; Magyar et al., 1980). The human fetal adrenal gland synthesizes cortisol de novo after the 28th week (beginning of third trimester) of pregnancy, which means the fetus is dependent on maternal cortisol for approximately the first 70% of pregnancy (Mastorakos and Ilias, 2003). In fetal sheep, the source of fetal cortisol is almost entirely maternal until GD 121 (the first 82% of pregnancy) and then, like the human, fetal adrenal responsiveness begins to change and fetal production increases. Fetal production is responsible for approximately 1/3 of the fetal cortisol concentration between GD 122–133 (Beitins et al.,

1970; Hennessy et al., 1982). After GD 133 in the sheep, the source of cortisol is almost exclusively of fetal origin. Likewise, in the human, cortisol levels triple late in pregnancy and undergo a further abrupt rise in association with labor (Murphy et al., 2006).

The findings from this study also help determine if, like alcohol, there are temporal windows of glucocorticoid effects on the brain, information that contributes to the search to elucidate the mechanisms of damage observed with different exposure periods across pregnancy. The timing of alcohol exposure during pregnancy is an important determinant of the type and extent of fetal brain damage that occurs (Clarren et al., 1978; Coles, 1994; Coles et al., 1991; Renwick and Asker, 1983; Riley et al., 2004) with both human and animal studies suggesting the developing cerebellum is vulnerable throughout gestation (Autti-Ramo et al., 2002; Bonthius and West, 1990; Goodlett et al., 1990a; Goodlett et al., 1990b; Hamre and West, 1993; Light et al., 2002b; Ramadoss et al., 2007a; Ramadoss et al., 2007b; West et al., 1990). Studies in the sheep and nonhuman primate models have reported significant reductions in cerebellar Purkinje cell counts regardless of whether alcohol exposure was during the period of brain development corresponding to the human first trimester, third trimester or all three trimesters (Bonthius et al., 1996; Ramadoss et al., 2007a; Ramadoss et al., 2007b). The stages of cerebellar development and thus mechanisms of Purkinje cell loss are markedly different between the first and third trimesters. The first trimester occurs prior to Purkinje cell genesis and so would affect precursor cells, whereas the third trimester exposure occurs after the genesis of Purkinje cells, during their post-mitotic differentiation. In sheep prenatal brain development, Purkinje cells have not been identified until after GD 80 (Rees and Harding, 1988; Salouci et al., 2012).

It has long been known that glucocorticoids play a critical role in normal development and appear to have organizational as well as regulatory effects, with evidence of regulation of cellular differentiation and neurotransmitter expression in various areas of the brain (Bohn, 1984; Rosenfeld et al., 1993). Glucocorticoid effects in the brain are mediated by two types of intracellular receptors: the glucocorticoid receptor (Type 2) and mineralocorticoid receptor (Type 1). The role of environmental factors in the long term regulation of receptor levels is not well established yet and is an area that remains under exploration (Rosenfeld et al., 1993). In vivo experiments provide more complete information than measurement of glucocorticoid receptor numbers in cell cultures because environmental effects and feedback mechanisms are intact. While receptors are necessary, they are not sufficient alone for glucocorticoids to exert their effects. Activities such as binding capacity of the receptor, ability to undergo transformation, binding to the nuclear response element, and alteration of transcription may all be subject to in vivo regulation. Testing in an intact organism and feto-maternal unit, as done in this study, allows for the presence and effect of these factors and thus helps elucidate physiologic significance.

Though no research has been done so far to define the ontogeny of glucocorticoid receptors in fetal cerebellar Purkinje cells using a pregnant sheep model, there are reports demonstrating the presence of glucocorticoid binding sites in fetal lamb pituitary, hypothalamic, and hippocampal tissues (Rose et al., 1985). Expression of glucocorticoid receptor mRNA in the developing sheep paraventricular nucleus has been demonstrated by GD 60, with maximum expression around GD 100–110 and in the developing sheep hippocampus and cerebral cortex, expression of glucocorticoid receptor mRNA was observed to be highest at GD 130 and GD 110 respectively (Andrews and Matthews, 2000). Ontogeny studies in the rat cerebellum reported intermediate levels of glucocorticoid receptor gene expression on postnatal day 0.5 (Speirs et al., 2004) and comparatively low levels of glucocorticoid induced receptors in rat Purkinje cells (Sah et al., 2005) which differs from the findings of Rosenfeld and coworkers showing evidence of high levels of glucocorticoid receptors in the rat cerebellum that remained unchanged with age (Rosenfeld

et al., 1993). Glucocorticoid receptor immunohistochemistry in the postnatal day 7 mouse showed light reactivity in the Purkinje cell layer (Noguchi et al., 2008). The affinity of glucocorticoid receptors for cortisol was also observed to be increase during the first week of postnatal life in the rat, which is equivalent to the human third trimester during which the brain growth spurt occurs (Dobbing and Sands, 1973, 1979; Rosenfeld et al., 1993) and is the developmental time period examined in this study.

Though this experimental design closely mimics the increases in cortisol seen in response to alcohol, there is a possibility that the fetal cortisol group and fetal alcohol groups did not have identical cortisol concentrations. This study utilized a third trimester equivalent exposure pattern in sheep (GD 109–133) and during this time, fetal adrenal responsiveness changes and approximately 1/3 of fetal cortisol is from fetal production on GD 121–133 (Beitins et al., 1970; Hennessy et al., 1982). Increases in maternal plasma cortisol concentration have been shown to increase fetal plasma cortisol levels and this in turn can provide negative feedback for fetal ACTH (Wood, 1987). Therefore during GD 121–133 it is possible that the increase in maternal plasma cortisol concentration in the cortisol group could have led to negative feedback in fetal ACTH levels, potentially lowering total fetal plasma cortisol concentration. This negative feedback would also occur in the alcohol group, but it has been demonstrated that alcohol also stimulates the fetal HPA axis (Cudd et al., 2001b) and it is possible that this could override the ACTH inhibition. Thus, the net result could be differences in fetal plasma cortisol concentrations in the alcohol and cortisol groups. The trending lower mean adrenal weight in the cortisol group suggests that they did indeed have high fetal plasma cortisol levels. Future experiments comparing prenatal steroid administration and alcohol exposure could be strengthened by measuring fetal cortisol and ACTH plasma concentrations, however this adds the potential confounding factor of surgery and instrumentation on cortisol concentrations since the earliest day fetal surgery can be performed is during the experimental period on GD 113.

In summary, our data demonstrates that repeated elevations in maternal glucocorticoids may not independently produce fetal cerebellar Purkinje cell loss during the brain growth spurt. However, the elevations in cortisol along with other changes induced by alcohol may together mediate brain injury in FASD.

Acknowledgments

Supported by NIAAA Grant AA10940 (TAC) and K08AA18166 (SEW).

References

Abbasi S, Hirsch D, Davis J, Tolosa J, Stouffer N, Debbs R, Gerdes JS. Effect of single versus multiple courses of antenatal corticosteroids on maternal and neonatal outcome. Am. J. Obstetr. Gyn. 2000; 182:1243–1249.

Andrews MH, Matthews SG. Regulation of glucocorticoid receptor mRNA and heat shock protein 70 mRNA in the developing sheep brain. Brain Res. 2000; 878:174–182. [PubMed: 10996148]

Autti-Ramo I, Autti T, Korkman M, Kettunen S, Salonen O, Valanne L. MRI findings in children with school problems who had been exposed prenatally to alcohol. Dev. Med. Child Neurol. 2002; 44:98–106. [PubMed: 11848116]

Beitins IZ, Kowarski A, Shermeta DW, De Lemos RA, Migeon CJ. Fetal and maternal secretion rate of cortisol in sheep: diffusion resistance of the placenta. Pediatric Res. 1970; 4:129–134.

Bodnoff SR, Humphreys AG, Lehman JC, Diamond DM, Rose GM, Meaney MJ. Enduring effects of chronic corticosterone treatment on spatial learning, synaptic plasticity, and hippocampal neuropathology in young and mid-aged rats. J. Neurosci. 1995; 15:61–69. [PubMed: 7823152]

Bohn, M. Glucocorticoid induced teratologies of the nervous system. Amsterdam/New York: Elsevier; 1984.

Bonthius DJ, Bonthius NE, Napper RM, Astley SJ, Clarren SK, West JR. Purkinje cell deficits in nonhuman primates following weekly exposure to ethanol during gestation. Teratology. 1996; 53:230–236. [PubMed: 8864164]

Bonthius DJ, West JR. Alcohol-induced neuronal loss in developing rats: increased brain damage with binge exposure. Alcohol. Clin. Exp. Res. 1990; 14:107–118. [PubMed: 1689970]

Caetano R, Ramisetty-Mikler S, Floyd LR, McGrath C. The epidemiology of drinking among women of child-bearing age. Alcoholism, clinical and experimental research. 2006; 30:1023–1030.

Clarren SK, Smith DW. The fetal alcohol syndrome. NEJM. 1978; 298:1063–1067. [PubMed: 347295]

Clarren SK, Alvord EC Jr, Sumi SM, Streissguth AP, Smith DW. Brain malformations related to prenatal exposure to ethanol. The Journal of pediatrics. 1978; 92:64–67. [PubMed: 619080]

Coles C. Critical periods for prenatal alcohol exposure. Alcohol Health Res. World. 1994; 18:22–29.

Coles CD, Brown RT, Smith IE, Platzman KA, Erickson S, Falek A. Effects of prenatal alcohol exposure at school ageIPhysical and cognitive development. Neurotoxicol. Tratol. 1991; 13:357–367.

Cragg B, Phillips S. Natural loss of Purkinje cells during development and increased loss with alcohol. Brain Res. 1985; 325:151–160. [PubMed: 4038892]

Cudd TA, Chen WJ, Parnell SE, West JR. Third trimester binge ethanol exposure results in fetal hypercapnea and acidemia but not hypoxemia in pregnant sheep. Alcohol. Clin. Exp. Res. 2001a; 25:269–276. [PubMed: 11236842]

Cudd TA, Chen WJ, West JR. Acute hemodynamic, pituitary, and adrenocortical responses to alcohol in adult female sheep. Alcohol. Clin. Exp. Res. 1996; 20:1675–1681. [PubMed: 8986221]

Cudd TA, Chen WJ, West JR. Fetal and maternal sheep hypothalamus pituitary adrenal axis responses to chronic binge ethanol exposure during the third trimester equivalent. Alcohol. Clin. Exp. Res. 2001b; 25:1065–1071. [PubMed: 11505034]

Dobbing J, Sands J. Quantitative growth and development of human brain. Arch. Dis. Child. 1973; 48:757–767. [PubMed: 4796010]

Dobbing J, Sands J. Comparative aspects of the brain growth spurt. Early Human Dev. 1979; 3:79–83.

Ebrahim SH, Diekman ST, Floyd RL, Decoufle P. Comparison of binge drinking among pregnant and nonpregnant women, United States, 1991–1995. Am. J. Obstetr. Gyn. 1999; 180:1–7.

Edwards HE, Burnham WM. The impact of corticosteroids on the developing animal. Pediatric Res. 2001; 50:433–440.

Goodlett CR, Eilers AT. Alcohol-induced Purkinje cell loss with a single binge exposure in neonatal rats: a stereological study of temporal windows of vulnerability. Alcohol. Clin. Exp. Res. 1997; 21:738–744. [PubMed: 9194933]

Goodlett CR, Hamre KM, West JR. Regional differences in the timing of dendritic outgrowth of Purkinje cells in the vermal cerebellum demonstrated by MAP2 immunocytochemistry. Brain Res. Dev. Brain Res. 1990a; 53:131–134.

Goodlett CR, Marcussen BL, West JR. A single day of alcohol exposure during the brain growth spurt induces brain weight restriction and cerebellar Purkinje cell loss. Alcohol. 1990b; 7:107–114. [PubMed: 2328083]

Goodlett CR, Pearlman AD, Lundahl KR. Binge neonatal alcohol intubations induce dose-dependent loss of Purkinje cells. Neurotoxicol. Teratol. 1998; 20:285–292. [PubMed: 9638686]

Gabriel K, Hofmann C, Glavas M, Weinberg J. The hormonal effects of alcohol use on the mother and fetus. Alcohol Health Res. World. 1998; 22:170–177. [PubMed: 15706792]

Gilstrap LC, Hightower ES, Clewell WH, D'Alton ME, Damon VG, Escobedo MB, Frader J, Gjerdingen DK, Goddard-Finegold J, Goldenberg RL, et al. Antenatal corticosteroids revisited: Repeat courses - National Institutes of Health consensus development conference statement, August 17–18, 2000. Obstetr. Gynecol. 2001; 98:144–150.

Gitau R, Cameron A, Fisk NM, Glover V. Fetal exposure to maternal cortisol. Lancet. 1998; 352:707–708. [PubMed: 9728994]

Gladstone J, Nulman I, Koren G. Reproductive risks of binge drinking during pregnancy. Reprod. Toxicol. 1996; 10:3–13. [PubMed: 8998383]

Gonzalez-Burgos I, Alejandre-Gomez M. Cerebellar granule cell and Bergmann glial cell maturation in the rat is disrupted by pre- and post-natal exposure to moderate levels of ethanol. Internat. J. Dev. Neurosci. 2005; 23:383–388.

Hamre KM, West JR. The effects of the timing of ethanol exposure during the brain growth spurt on the number of cerebellar Purkinje and granule cell nuclear profiles. Alcohol. Clin. Exp. Res. 1993; 17:610–622. [PubMed: 8333592]

Hennessy DP, Coghlan JP, Hardy KJ, Scoggins BA, Wintour EM. The origin of cortisol in the blood of fetal sheep. J. Endocrinol. 1982; 95:71–79. [PubMed: 7130892]

Hoyme HE, May PA, Kalberg WO, Kodituwakku P, Gossage JP, Trujillo PM, Buckley DG, Miller JH, Aragon AS, Khaole N, et al. A practical clinical approach to diagnosis of fetal alcohol spectrum disorders: clarification of the 1996 institute of medicine criteria. Pediatrics. 2005; 115:39–47. [PubMed: 15629980]

Huang WL, Beazley LD, Quinlivan JA, Evans SF, Newnham JP, Dunlop SA. Effect of corticosteroids on brain growth in fetal sheep. Obstetr. Gynecol. 1999; 94:213–218.

Huang WL, Harper CG, Evans SF, Newnham JP, Dunlop SA. Repeated prenatal corticosteroid administration delays astrocyte and capillary tight junction maturation in fetal sheep. Internat. J. Dev. Neurosci. 2001a; 19:487–493.

Huang WL, Harper CG, Evans SF, Newnham JP, Dunlop SA. Repeated prenatal corticosteroid administration delays myelination of the corpus callosum in fetal sheep. Internat. J. Dev. Neurosci. 2001b; 19:415–425.

Institute, oM. Diagnosis, epidemiology, Prevention, and Treatment. Washington DC: Institute of Medicine; 1996. Fetal alcohol Syndrome.

Jacobson SW, Bihun JT, Chiodo LM. Effects of prenatal alcohol and cocaine exposure on infant cortisol levels. Devel. Psychopathol. 1999; 11:195–208. [PubMed: 16506530]

Jameson RR, Seidler FJ, Qiao D, Slotkin TA. Adverse neurodevelopmental effects of dexamethasone modeled in PC12 cells: identifying the critical stages and concentration thresholds for the targeting of cell acquisition, differentiation and viability. Neuropsychopharmacology. 2006; 31:1647–1658. [PubMed: 16319912]

Jensen E, Wood CE, Keller-Wood M. Chronic alterations in ovine maternal corticosteroid levels influence uterine blood flow and placental and fetal growth. Am. J. Physiol. Regul. Integr. Comp. Physiol. 2005; 288:R54–R61. [PubMed: 15231491]

Jobe AH, Wada N, Berry LM, Ikegami M, Ervin MG. Single and repetitive maternal glucocorticoid exposures reduce fetal growth in sheep. Am. J. Obstetr. Gynecol. 1998; 178:880–885.

Jones KL, Smith DW. Recognition of the fetal alcohol syndrome in early infancy. Lancet. 1973; 302:999–1001. [PubMed: 4127281]

Jones KL, Smith DW, Streissguth AP, Myrianthopoulos NC. Outcome in offspring of chronic alcoholic women. Lancet. 1974; 1:1076–1078. [PubMed: 4135246]

Jones KL, Smith DW, Ulleland CN, Streissguth P. Pattern of malformation in offspring of chronic alcoholic mothers. Lancet. 1973; 1:1267–1271. [PubMed: 4126070]

Kakihana R, Butte JC, Moore JA. Endocrine effects of meternal alcoholization: plasma and brain testosterone, dihydrotestosterone, estradiol, and corticosterone. Alcohol. Clin. Exp. Res. 1980; 4:57–61. [PubMed: 6986816]

Kim CK, Giberson PK, Yu W, Zoeller RT, Weinberg J. Effects of prenatal ethanol exposure on hypothalamic-pituitary-adrenal responses to chronic cold stress in rats. Alcohol. Clin. Exp. Res. 1999; 23:301–310. [PubMed: 10069560]

Kutzler MA, Ruane EK, Coksaygan T, Vincent SE, Nathanielsz PW. Effects of three courses of maternally administered dexamethasone at 0.7, 0.75, and 0.8 of gestation on prenatal and postnatal growth in sheep. Pediatrics. 2004; 113:313–319. [PubMed: 14754943]

Lemoine P, Harousseau H, Borteyru JP, Menuet JC. Children of alcoholic parents--observed anomalies: discussion of 127 cases. Therapeut. Drug Monitor. 2003; 25:132–136.

Light KE, Belcher SM, Pierce DR. Time course and manner of Purkinje neuron death following a single ethanol exposure on postnatal day 4 in the developing rat. Neuroscience. 2002a; 114:327–337. [PubMed: 12204202]

Light KE, Brown DP, Newton BW, Belcher SM, Kane CJ. Ethanol-induced alterations of neurotrophin receptor expression on Purkinje cells in the neonatal rat cerebellum. Brain Res. 2002b; 924:71–81. [PubMed: 11743997]

Lupton C, Burd L, Harwood R. Cost of fetal alcohol spectrum disorders. Am. J. Med. Genet. C Semin. Med. Genet. 2004; 127C:42–50. [PubMed: 15095471]

Magyar DM, Fridshal D, Elsner CW, Glatz T, Eliot J, Klein AH, Lowe KC, Buster JE, Nathanielsz PW. Time-trend analysis of plasma cortisol concentrations in the fetal sheep in relation to parturition. Endocrinology. 1980; 107:155–159. [PubMed: 7379742]

Maier SE, Miller JA, Blackwell JM, West JR. Fetal alcohol exposure and temporal vulnerability: regional differences in cell loss as a function of the timing of binge-like alcohol exposure during brain development. Alcohol. Clin. Exp. Res. 1999; 23:726–734. [PubMed: 10235310]

Maier SE, West JR. Regional differences in cell loss associated with binge-like alcohol exposure during the first two trimesters equivalent in the rat. Alcohol. 2001; 23:49–57. [PubMed: 11282452]

Maloney SE, Noguchi KK, Wozniak DF, Fowler SC, Farber NB. Long-term Effects of Multiple Glucocorticoid Exposures in Neonatal Mice. Behav. Sci. 2011; 1:4–30.

Mastorakos G, Ilias I. Maternal and fetal hypothalamic-pituitary-adrenal axes during pregnancy and postpartum. Annals NY Acad. Sci. 2003; 997:136–149.

May PA, Gossage JP. Estimating the prevalence of fetal alcohol syndrome. A summary. Alcohol Res. Health. 2001; 25:159–167. [PubMed: 11810953]

McGowan JE, Sysyn G, Petersson KH, Sadowska GB, Mishra OP, Delivoria-Papadopoulos M, Stonestreet BS. Effect of dexamethasone treatment on maturational changes in the NMDA receptor in sheep brain. J. Neurosci. 2000; 20:7424–7429. [PubMed: 11007901]

Miller SL, Chai M, Loose J, Castillo-Melendez M, Walker DW, Jenkin G, Wallace EM. The effects of maternal betamethasone administration on the intrauterine growth-restricted fetus. Endocrinology. 2007; 148:1288–1295. [PubMed: 17158204]

Milley JR. Effects of increased cortisol concentration on ovine fetal leucine kinetics and protein metabolism. Am. J. Physiol. 1995; 268:E1114–E1122. [PubMed: 7611386]

Modi N, Lewis H, Al-Naqeeb N, Ajayi-Obe M, Dore CJ, Rutherford M. The effects of repeated antenatal glucocorticoid therapy on the developing brain. Pediatric Res. 2001; 50:581–585.

Murphy BP, Inder TE, Huppi PS, Warfield S, Zientara GP, Kikinis R, Jolesz FA, Volpe JJ. Impaired cerebral cortical gray matter growth after treatment with dexamethasone for neonatal chronic lung disease. Pediatrics. 2001; 107:217–221. [PubMed: 11158449]

Murphy VE, Smith R, Giles WB, Clifton VL. Endocrine regulation of human fetal growth: the role of the mother, placenta, and fetus. Endocrine Rev. 2006; 27:141–169. [PubMed: 16434511]

Newnham JP, Evans SF, Godfrey M, Huang W, Ikegami M, Jobe A. Maternal, but not fetal, administration of corticosteroids restricts fetal growth. J. Maternal-Fetal Med. 1999; 8:81–87.

NIH CDP. Effect of corticosteroids for fetal maturation on perinatal outcomes. NIH Consensus Development Panel on the Effect of Corticosteroids for Fetal Maturation on Perinatal Outcomes. JAMA. 1995; 273:413–418. [PubMed: 7823388]

Noguchi KK, Walls KC, Wozniak DF, Olney JW, Roth KA, Farber NB. Acute neonatal glucocorticoid exposure produces selective and rapid cerebellar neural progenitor cell apoptotic death. Cell Death Differentiation. 2008; 15:1582–1592. [PubMed: 18600230]

Pauli J, Wilce P, Bedi KS. Acute exposure to alcohol during early postnatal life causes a deficit in the total number of cerebellar Purkinje cells in the rat. J. Comp. Neurology. 1995; 360:506–512.

Penton Z. Headspace measurement of ethanol in blood by gas chromatography with a modified autosampler. Clin. Chem. 1985; 31:439–441. [PubMed: 3971565]

Ramadoss J, Lunde ER, Chen WJ, West JR, Cudd TA. Temporal vulnerability of fetal cerebellar Purkinje cells to chronic binge alcohol exposure: ovine model. Alcohol. Clin. Exp. Res. 2007a; 31:1738–1745. [PubMed: 17681031]

Ramadoss J, Lunde ER, Pina KB, Chen WJ, Cudd TA. 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. 2007b; 31:1252–1258. [PubMed: 17511745]

Ramadoss J, Tress U, Chen WJ, Cudd TA. Maternal adrenocorticotropin, cortisol, and thyroid hormone responses to all three-trimester equivalent repeated binge alcohol exposure: ovine model. Alcohol. 2008; 42:199–205. [PubMed: 18420114]

Ramadoss J, Lunde ER, Ouyang N, Chen WJ, Cudd TA. Acid-sensitive channel inhibition prevents fetal alcohol spectrum disorders cerebellar Purkinje cell loss. Am.J. Physiol. Regul. Integr. Comp. Physiol. 2008; 295:R596–R603. [PubMed: 18509098]

Rees S, Harding R. The effects of intrauterine growth retardation on the development of the Purkinje cell dendritic tree in the cerebellar cortex of fetal sheep: a note on the ontogeny of the Purkinje cell. Intern. J. Dev. Neurosci. 1988; 6:461–469.

Renwick JH, Asker RL. Ethanol-sensitive times for the human conceptus. Early Hum. Devel. 1983:99–111. [PubMed: 6884260]

Riley EP, McGee CL, Sowell ER. Teratogenic effects of alcohol: a decade of brain imaging. Am. J. Med. Genet. C Semin. Med. Genet. 2004; 127C:35–41. [PubMed: 15095470]

Rose JC, Kute TE, Winkler L. Glucocorticoid receptors in sheep brain tissues during development. Am. J. Physiol. 1985; 249:E345–E349. [PubMed: 4050987]

Rosenfeld P, van Eekelen JA, Levine S, de Kloet ER. Ontogeny of corticosteroid receptors in the brain. Cell. Mol. Neurobiol. 1993; 13:295–319. [PubMed: 8252605]

Rosett HL. A clinical perspective of the Fetal Alcohol Syndrome. Alcohol. Clin. Exp. Res. 1980; 4:119–122. [PubMed: 6990814]

Rugerio-Vargas C, Ramirez-Escoto M, DelaRosa-Rugerio C, Rivas-Manzano P. Prenatal corticosterone influences the trajectory of neuronal development, delaying or accelerating aspects of the Purkinje cell differentiation. Histol. Histopathol. 2007; 22:963–969. [PubMed: 17523073]

Sah R, Pritchard LM, Richtand NM, Ahlbrand R, Eaton K, Sallee FR, Herman JP. Expression of the glucocorticoid-induced receptor mRNA in rat brain. Neuroscience. 2005; 133:281–292. [PubMed: 15893650]

Salouci M, Engelen V, Gyan M, Antoine N, Jacqmot O, Mignon Y, Kirschvink N, Gabriel A. Development of Purkinje cells in the ovine brain. Anatomia Histologia Embryologia. 2012; 41:227–232.

Schneider ML, Moore CF, Kraemer GW, Roberts AD, DeJesus OT. The impact of prenatal stress, fetal alcohol exposure, or both on development: perspectives from a primate model. Psychoneuroendocrinology. 2002; 27:285–298. [PubMed: 11750784]

Sokol RJ, Clarren SK. Guidelines for use of terminology describing the impact of prenatal alcohol on the offspring. Alcohol. Clin. Exp. Res. 1989; 13:597–598. [PubMed: 2679217]

Sokol RJ, Delaney-Black V, Nordstrom B. Fetal alcohol spectrum disorder. JAMA. 2003; 290:2996–2999. [PubMed: 14665662]

Speirs HJ, Seckl JR, Brown RW. Ontogeny of glucocorticoid receptor and 11betahydroxysteroid dehydrogenase type-1 gene expression identifies potential critical periods of glucocorticoid susceptibility during development. J. Endocrinol. 2004; 181:105–116. [PubMed: 15072571]

Velazquez PN, Romano MC. Corticosterone therapy during gestation: effects on the development of rat cerebellum. Inter. J. devel. Neurosci. 1987; 5:189–194.

Weinberg J. Prenatal ethanol exposure alters adrenocortical development of offspring. Alcohol. Clin. Exp. Res. 1989; 13:73–83. [PubMed: 2538092]

West JR, Parnell SE, Chen WJ, Cudd TA. Alcohol-mediated Purkinje cell loss in the absence of hypoxemia during the third trimester in an ovine model system. Alcohol. Clin. Exp. Res. 2001; 25:1051–1057. [PubMed: 11505032]

West JR, Goodlett CR, Bonthius DJ, Hamre KM, Marcussen BL. Cell population depletion associated with fetal alcohol brain damage: mechanisms of BAC-dependent cell loss. Alcohol. Clin. Exp. Res. 1990; 14:813–818. [PubMed: 2088116]

Wilson SE, Cudd TA. Focus on: The use of animal model for the study of fetal alcohol spectrum disorders. Alcohol Res. Health. 2011; 34:92–98. [PubMed: 23580046]

Wood CE. Negative-feedback inhibition of fetal ACTH secretion by maternal cortisol. Am. J. Physiol. 1987; 252:R743–R748. [PubMed: 3032007]

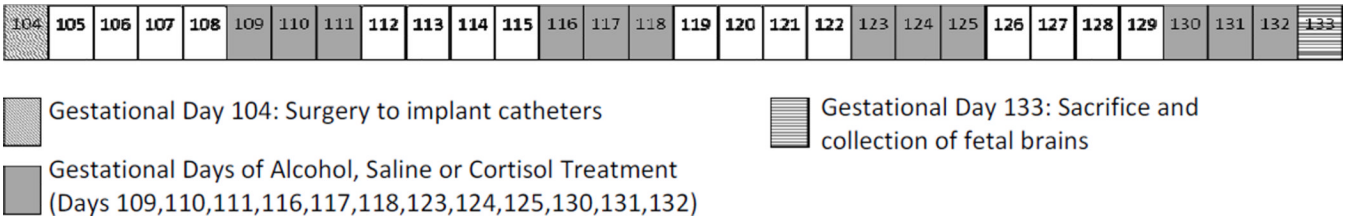

Figure 1.

Experimental treatment paradigm. Infusions were administered on three consecutive days followed by four days without alcohol beginning on gestational day (GD) 109 and continuing until GD132. Surgery to implant vascular catheters was performed on GD 104. On GD 133, animals were sacrificed and fetal brains were collected.

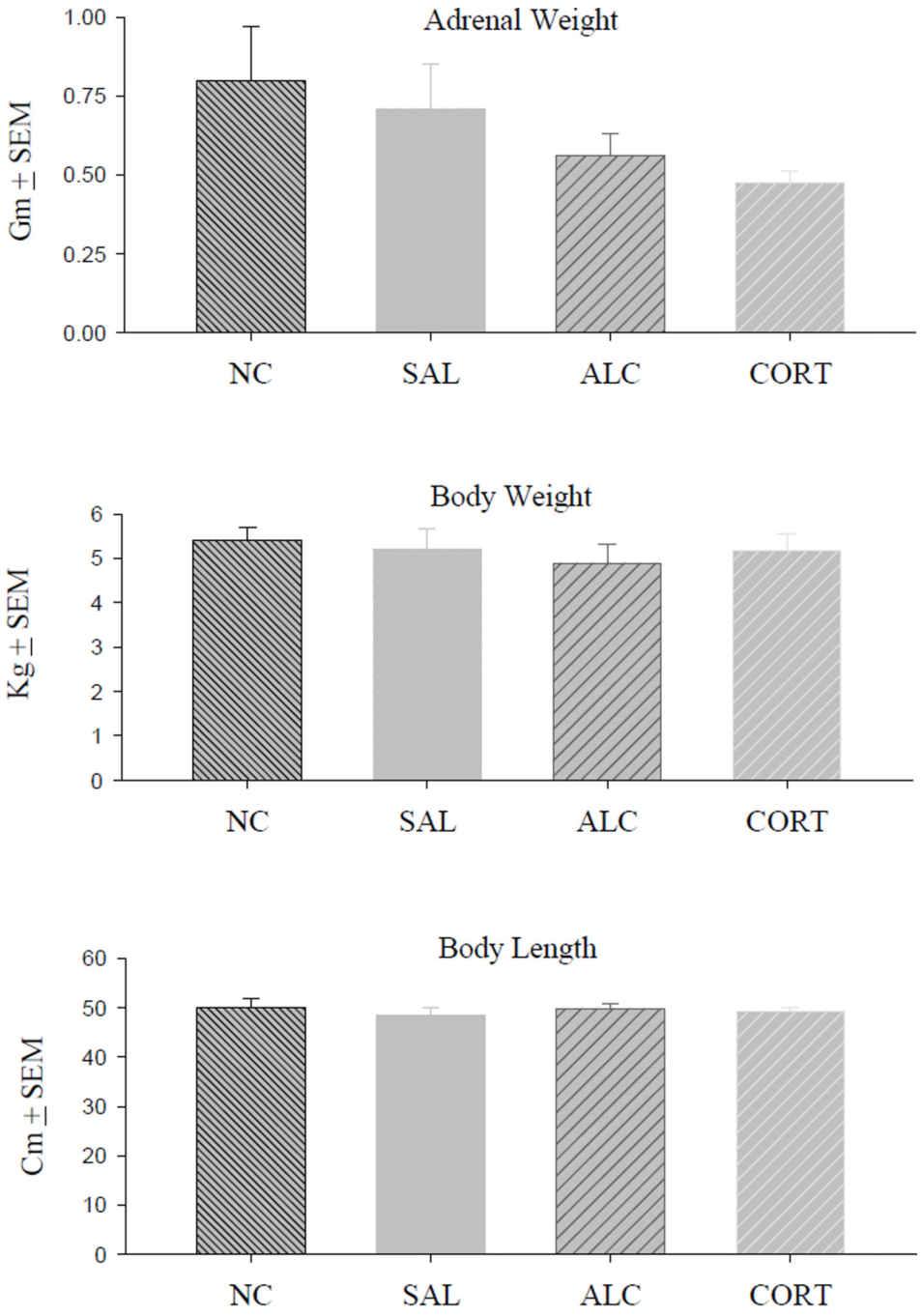

Figure 2.

Fetal growth measures in normal control (NC), saline (SAL), alcohol (ALC) and cortisol (CORT) groups. Fetal whole body weight and body length (crown to rump) were not different among groups. Fetal adrenal weight was not statistically different between groups (p=0.08).

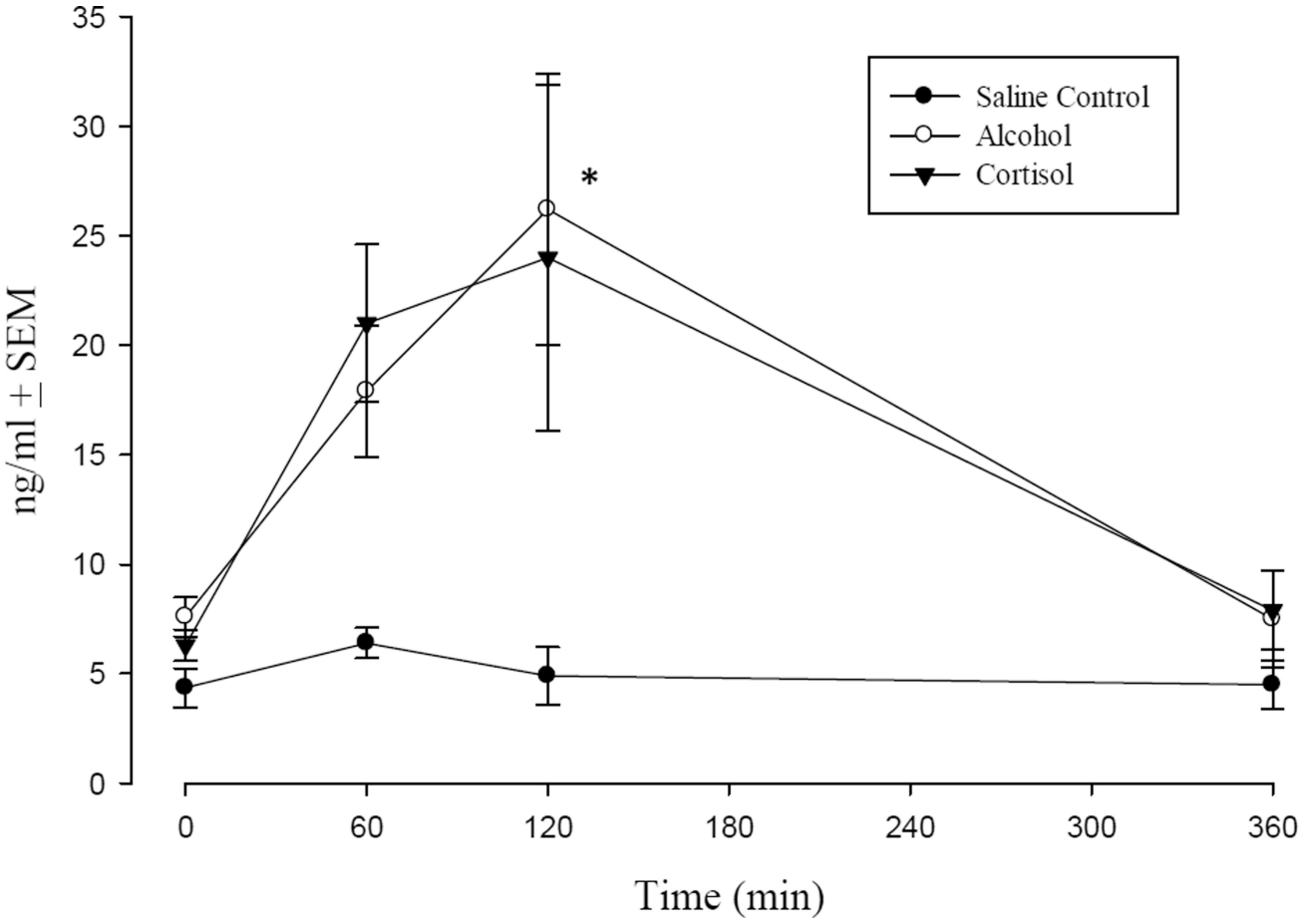

Figure 3.
Maternal plasma cortisol levels (from experiment on gestational day 132). The magnitude of elevation in cortisol in response to alcohol was mimicked in the cortisol group by infusing pregnant ewes with hydrocortisone for 6 hours on each day of the experiment. The cortisol and alcohol groups were not significantly different from each other at any time point, but both groups were significantly increased at 120 minutes compared to the saline control group (*). Maternal cortisol levels were not significantly altered in the saline control group at any time point.

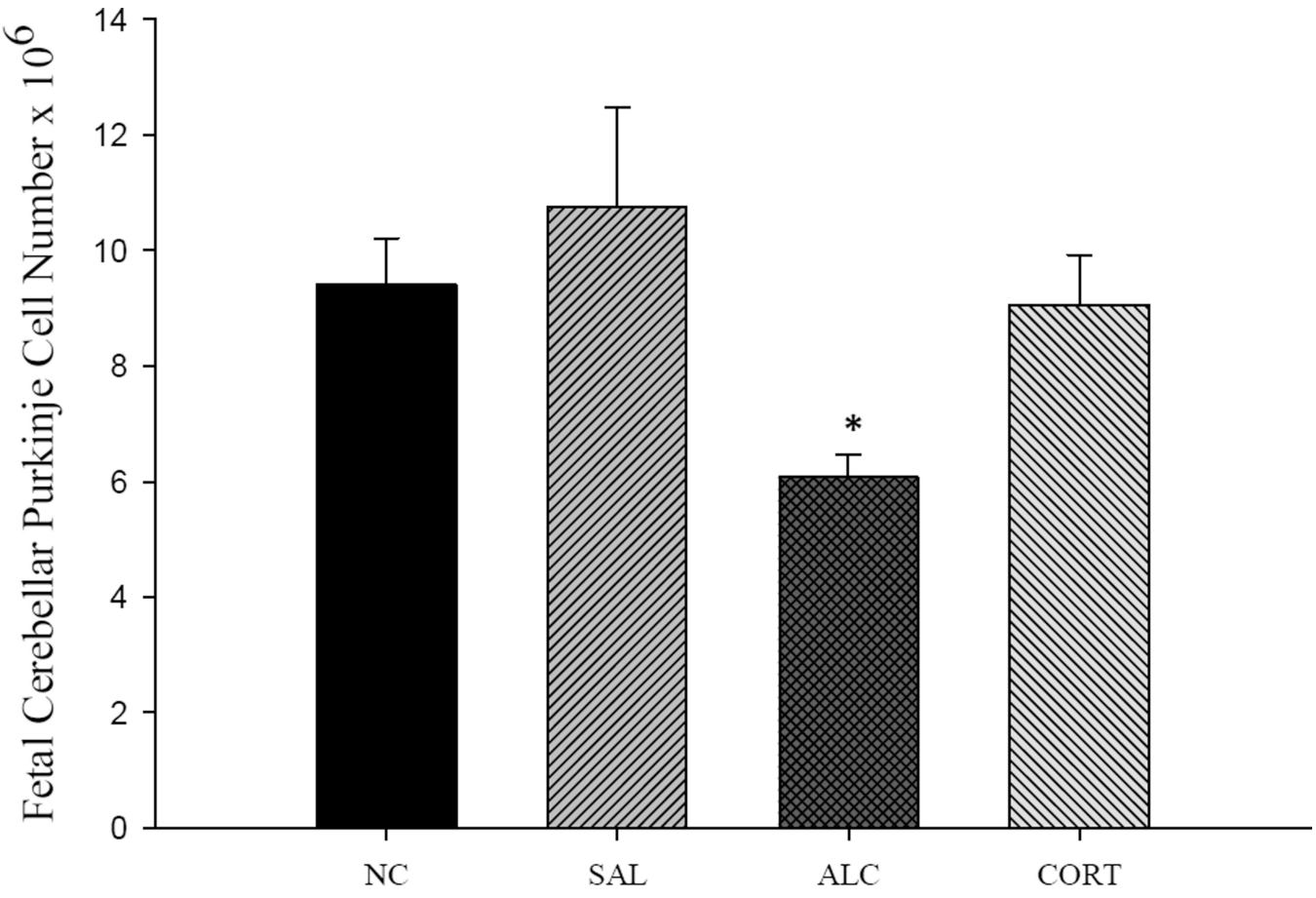

Figure 4.
Estimated fetal cerebellar Purkinje cell number (× 10^6) in normal control (NC), saline (SAL), alcohol (ALC) and cortisol (CORT) groups. Alcohol exposure significantly decreased total Purkinje cell number compared to the normal control, saline control, and the cortisol treatment groups (*). No differences were noted between the controls and the cortisol group.

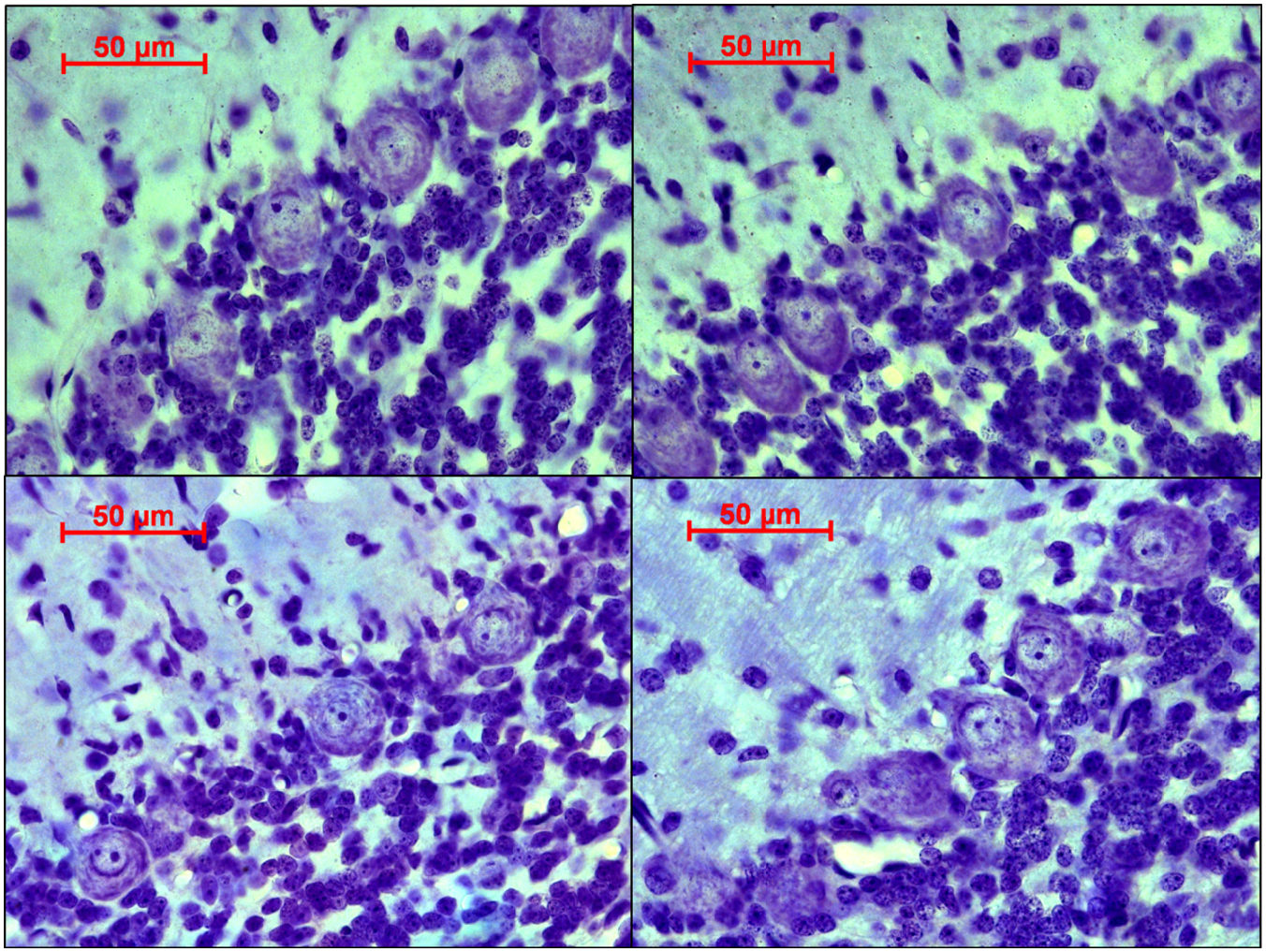

Figure 5.
Histological images from each treatment group. A is from the normal control group, B from the saline group, C from the alcohol group, and D from the cortisol group.