Maternal choline supplementation in a sheep model of first trimester binge alcohol fails to protect against brain volume reductions in peripubertal lambs

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Abstract

Fetal alcohol spectrum disorder (FASD) is a leading potentially preventable birth defect. Poor nutrition may contribute to adverse developmental outcomes of prenatal alcohol exposure, and supplementation of essential micronutrients such as choline has shown benefit in rodent models.

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The sheep model of first-trimester binge alcohol exposure was used in this study to model the dose of maternal choline supplementation used in an ongoing prospective clinical trial involving pregnancies at risk for FASD. Primary outcome measures included volumetrics of the whole brain, cerebellum, and pituitary derived from magnetic resonance imaging (MRI) in 6-month-old lambs, testing the hypothesis that alcohol-exposed lambs would have brain volume reductions that would be ameliorated by maternal choline supplementation. Pregnant sheep were randomly assigned to one of five groups – heavy binge alcohol (HBA; 2.5 g/kg/treatment ethanol), heavy binge alcohol plus choline supplementation (HBC; 2.5 g/kg/treatment ethanol and 10 mg/kg/day choline), saline control (SC), saline control plus choline supplementation (SCC; 10 mg/kg/day choline), and normal control (NC). Ewes were given intravenous alcohol (HBA, HBC; mean peak BACs of ~280 mg/dL) or saline (SC, SCC) on three consecutive days per week from gestation day (GD) 4–41; choline was administered on GD 4–148. MRI scans of lamb brains were performed postnatally on day 182. Lambs from both alcohol groups (with or without choline) showed significant reductions in total brain volume; cerebellar and pituitary volumes were not significantly affected. This is the first report of MRI-derived volumetric brain reductions in a sheep model of FASD following binge-like alcohol exposure during the first trimester. These results also indicate that maternal choline supplementation comparable to doses in human studies fails to prevent brain volume reductions typically induced by first-trimester binge alcohol exposure. Future analyses will assess behavioral outcomes along with regional brain and neurohistological measures.

Keywords

choline; volumetrics; prenatal alcohol; FASD; diagnosis; magnetic resonance imaging

Introduction

Prenatal alcohol exposure results in multiple teratogenic effects on central nervous system (CNS) development, including altered cellular proliferation and migration, decreased synaptic connectivity, increased apoptotic cell death, and impaired myelination. These may all contribute to brain growth restriction, neurodevelopmental delays, changes in structure and function, and resultant abnormal behavior and cognition (Goodlett, Horn, & Zhou, 2005; Guerri, Bazinet, & Riley, 2009; Ismail, Buckley, Budacki, Jabbar, & Gallicano, 2010; Pollard, 2007; Thompson, Levitt, & Stanwood, 2009). The presence of CNS abnormalities, together with dysmorphic facial features and growth deficits, constitute the three characteristic phenotypes required to diagnose fetal alcohol syndrome (FAS) (Bertrand, Floyd, & Weber, 2005; Wattendorf & Muenke, 2005). However, the majority of children expressing adverse cognitive and neurodevelopmental effects resulting from prenatal alcohol exposure do not meet the diagnostic criteria for FAS. An umbrella classification, fetal alcohol spectrum disorders (FASD), has been adopted to encompass the full spectrum of alcohol-related neurodevelopmental disorders (Riley & McGee, 2005; Suttie et al., 2013; Ware et al., 2013).

Given that FASD is a leading potentially preventable birth defect (NIAAA, 2012), with prevalence estimates ranging from 1% (Hoyme et al., 2005; Leibson, Neuman, Chudley, & Koren, 2014) to as high as 2–5% (May et al., 2009), priorities have been placed on efforts to

develop early interventions that may provide significant benefits and thus mitigate the lifelong effects of FASD. Choline supplementation has emerged as a particularly promising nutraceutical therapeutic approach, based mainly on positive results from preclinical rodent models (Idrus & Thomas, 2011).

Choline is classified as an essential nutrient that has many important roles in brain development and function. It is involved directly as a precursor or through metabolites in maintaining the integrity of cell membranes and cell signaling, lipid and cholesterol transport, synthesis of the neurotransmitter acetylcholine, methyl metabolism, and DNA methylation (Ballard, Sun, & Ko, 2012; Zeisel & da Costa, 2009). The recommended daily intake of choline for pregnant women is 450 mg/day (Institute of Medicine [US] Standing Committee on the Scientific Evaluation of Dietary Reference and its Panel on Folate, Other B Vitamins, and Choline, 1998). During pregnancy, phosphatidylcholine production is increased in the liver, providing an important source of choline to the developing fetus (Zeisel, 2011). Choline deficiency has been implicated in the molecular etiology of FASD because alcohol can decrease choline availability to the fetus by several mechanisms. The detrimental effects of choline deficiency may be further exacerbated by reduced maternal intake of choline and thus fetal deficiency of choline can ultimately contribute to abnormal brain development (Shaw et al., 2009; Zeisel, 2011).

Studies with rodent models of FASD over the previous 15 years by Thomas and colleagues as well as by others have demonstrated that choline administration during and/or after the time of alcohol exposure (either during gestation or during the early neonatal period of brain development in rats comparable to that of the human third trimester) can significantly ameliorate a variety of alcohol-induced neurobehavioral deficits. Choline supplementation has been shown to ameliorate deficits in behavioral and physical development and brain weight (Thomas, Abou, & Dominguez, 2009), spatial learning and memory (Ryan, Williams, & Thomas, 2008; Thomas, Biane, O'Bryan, O'Neill, & Dominguez, 2007), spatial working memory (Thomas, Idrus, Monk, & Dominguez, 2010), visual discrimination (Thomas, La Fiette, Quinn, & Riley, 2000), locomotor hyperactivity (Monk, Leslie, & Thomas, 2012; Thomas, Garrison, & O'Neill, 2004), trace fear conditioning (Wagner & Hunt, 2006), and trace eyeblink conditioning (Thomas & Tran, 2012). Positive improvements associated with choline supplementation in the rodent models were achieved with daily doses (typically 250 mg/kg/day) that far exceed the minimum daily choline requirements for rodents.

Based on these promising results from the rodent models, the Collaborative Initiative on Fetal Alcohol Spectrum Disorders (CIFASD) designed and implemented a prospective randomized clinical trial in the Ukraine involving multivitamin and mineral (MVM) supplements in which a subgroup of the MVM group was also given a choline supplement (750 mg/day, about 10 mg/kg/day) as an additional component of the MVM intervention. The sheep model study reported here was concurrently designed to optimize the translational comparisons with the prospective randomized human clinical trial. A choline dosing regimen of 10 mg/kg/day was selected and the choline supplement was started at the beginning of pregnancy (at the start of the binge-alcohol exposure period), whereas in clinical trials, participants were enrolled after the first prenatal appointment (typically

around 19 weeks gestation). Choline supplementation was initiated at the start of alcohol exposure in the current study to maximize the likelihood of benefits from early intervention. This is the first report of a sheep model with choline supplementation, which models the CIFASD Ukrainian clinical trial that was recently published (Coles et al., 2015).

Although rodent studies have provided the primary foundation of research in FASD, including the use of choline as a potential intervention, the sheep model provides important (if not unique) translational value. The gestational period in sheep is relatively long (147 days) with the first-trimester human equivalent extending from GD 4–41, the second-trimester equivalent from GD 42–108, and the third-trimester equivalent from GD 109–132 (Sawant et al., 2013). Sheep also serve as a better model for humans in terms of the rate of brain development over gestation (the third-trimester equivalent takes place *in utero* similarly to humans and in contrast to the rodent model), the body and brain mass of the fetus, and maternal-fetal physiology. Techniques for instrumentation and experimental manipulation of the pregnant ewe are also now well established and feasible.

The primary goal of this study was to model binge drinking in the first trimester and evaluate maternal choline supplementation administered throughout gestation as a preventative intervention for pregnancies at risk for FASD. We used a heavy binge-like alcohol treatment to model repeated weekend binge drinking over the first-trimester equivalent (GD 4–41), a pattern of drinking which is similar in women who abuse alcohol during pregnancy and a known risk factor for FASD (Conover & Jones, 2012; Maier & West, 2001; Ramadoss, Hogan, Given, West, & Cudd, 2006). A second goal of this study was to incorporate structural volumetrics with MRI to obtain brain volume measures like those done in human studies. Although the use of MRI is routine in clinical veterinary medicine, quantitative 3-dimensional brain imaging for experimental studies has not been developed for use in sheep or other large animals. Therefore many of the tools for automated segmentation and quantification of various brain regions, commonly available in humans and rodents (Denic et al., 2011), have not been developed for experimental veterinary applications. For this study, protocols and procedures were developed so that quantitative data from the sheep brain could be compared to measures routinely available from structural imaging in children with FASD (Moore, Migliorini, Infante, & Riley, 2014). Volumetric reductions have been demonstrated in the whole brain (De Guio et al., 2014; Rajaprakash, Chakravarty, Lerch, & Rovet, 2014; Yang et al., 2012) and cerebellum (Archibald et al., 2001; de Zeeuw, Zwart, Schrama, van Engeland, & Durston, 2012; O'Hare et al., 2005; Riikonen, Salonen, Partanen, & Verho, 1999) and many studies have documented more selective changes in specific brain regions (Donald et al., 2015; Spadoni, McGee, Fryer, & Riley, 2007).

Given our goal of implementing structural MRI protocols in the sheep model, our first approach was to obtain quantitative volumetric data of the whole brain of peripubertal lambs (6 months old), a stage roughly equivalent to children entering puberty during early adolescence (*The Merck Veterinary Manual*, 2005). The cerebellum was also evaluated because it is easily segmented and it is known to be vulnerable to prenatal alcohol exposure (Bookstein, Streissguth, Connor, & Sampson, 2006; Cardenas et al., 2014; de Zeeuw et al., 2012; O'Hare et al., 2005; Sowell et al., 2002). We also quantified the volume of the pituitary as a potentially contrasting outcome, given the interesting findings reported in

mouse models of binge-like gestational exposure. In these studies, exposure either on gestational day 8 or on gestational days 12–16 yielded increased pituitary volume when assessed on gestational day 17 (Parnell, Holloway, Baker, Styner, & Sulik, 2014; Parnell et al., 2009). Our fundamental hypothesis was that prenatal binge-like alcohol exposure over the first-trimester equivalent would produce brain volume reductions detectable by MRI, directly modeling the microencephaly seen in children with FAS. We also tested the hypothesis that maternal supplementation of choline in doses similar to those used in the CIFASD clinical trial, administered throughout the entire pregnancy, would protect against brain volume reductions.

Materials and methods

Animals

All animals and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at Texas A&M University. Prior to breeding, Suffolk ewes (aged 2–5 years) received multi-species Clostridium bacterin-toxoid (Covexin 8, Intervet/Schering-Plough Animal Health, Summit, NJ) 2 mL intramuscularly, albendazole (Valbazen Suspension 7.5 mg/kg, Pfizer Animal Health, New York, NY) 0.75 mL/25 lb. of body weight orally, and ivermectin (Ivomec Drench for Sheep 0.8%, Merial, Duluth, GA) 3 mL/26 lb. body weight orally. The day of mating, corresponding to when ewes were marked by the ram, was designated as GD 0. Ewes were penned individually but had visual contact at all times with herd mates in adjacent pens in an environmentally regulated facility (22 °C and a 12:12-h light/dark cycle). Pregnancy was confirmed ultrasonographically on GD 25 and ewes that were not pregnant were removed from the experiment. Ewes were fed a "complete" ration (TAMU Ewe Ration, Nutrena, Cargill, Minneapolis, MN) designed to meet 100% National Research Council requirements (feed does not include choline) as calculated by ARIES (software version 2007, University of California, Davis) and had free access to drinking water. Maternal food consumption was monitored daily. All subjects consumed all feed offered.

Choline dose development

The aim of this study was to model the choline dose implemented in the CIFASD clinical trial, thus providing a strong basis for translational comparisons. This study intentionally avoided the very high doses of choline used in the rodent models (~250 mg/kg per day) because they are ~25× higher (on a per kg basis) than the doses used in the Ukraine clinical trial (Coles et al., 2015). The high choline doses often used in rodent studies are not likely to be used in clinical studies in pregnant women. The choline dose we chose (10 mg/kg per day) is higher than the current daily-recommended dose during human pregnancy (450 mg/day, ~6 mg/kg per day). In the CIFASD randomized clinical trial, alcohol-using and nondrinking women were randomized to one of three multivitamin/mineral supplement groups: none, multivitamins/minerals (MVM), and multivitamin/minerals plus choline. The choline group was given a daily dose of 750 mg (~10 mg/kg per day), thus helping to guide the decision for the dose of choline used in the sheep model (Coles et al., 2015). Higher daily doses of choline (on a mg/kg basis) may be given postnatally in humans (Wozniak et al., 2013), but this sheep model was developed to test choline supplementation during

pregnancy with binge-like alcohol exposure during the first-trimester equivalent. Consequently, the dosing regimen used in this sheep model is both relevant and highly translational in respect to dose of choline and timing outcome of the children postnatally.

In ruminants, choline is absorbed maximally in the abomasum and small intestine but it is often degraded in the rumen on its way. Because of this, a commercially available microencapsulated choline supplement (designed for ruminants, primarily dairy cattle) was developed to allow the choline supplement to survive transfer through the rumen where it can be absorbed in the abomasum and small intestine (ReaShure® [choline chloride 28.8%], Balchem Corporation, New Hampton, NY). To test this product in sheep, we fed 10 mg/kg of choline (based on weight of choline in ReaShure®) to 10 non-pregnant ewes and then collected abomasal contents 30, 60, and 120 min after administration. Choline levels in these samples were compared to the abomasal contents from 6 normal control animals that did not receive a choline supplement. The level of choline in the abomasum was significantly higher in both the 30-min (848 nmol/mL) and 60-min (627 nmol/mL) samples following choline administration with ReaShure® as compared to the normal controls (557 nmol/mL) ($p < 0.001$). Abomasal levels returned to baseline levels comparable to the normal controls at the 120 min time point.

In a second preliminary study to evaluate plasma choline and betaine levels, 14 pregnant ewes were assigned to three treatment groups: 1) saline control choline (SCC; isotonic saline [0.9%] during the 1st trimester plus 10 mg/kg/day choline until GD 133), 2) heavy binge alcohol (HBA; 2.5 g/kg/treatment ethanol during the 1st trimester), and 3) heavy binge alcohol plus choline (HBC; 2.5 g/kg/treatment ethanol during 1st trimester plus 10 mg/kg/day choline until GD 133). Plasma samples were collected on GD 134 and shipped frozen to the laboratory of Dr. C. Keen at University of California-Davis, who performed the choline analyses using mass spectroscopy. Due to the limited number of available ewes, no saline control (SC; no choline) group was included for the GD 134 samples. However, a set of four normal control (NC) ewes were sampled on GD 147 to provide a comparative index of choline levels during late pregnancy. For the GD 134 samples, plasma choline levels were significantly higher ($p < 0.05$) in the two choline-treated groups, SCC (5.55 ± 0.03 μM) and HBC (6.08 ± 0.62 μM), compared to the alcohol-only group, HBA (3.94 ± 0.15 μM). Betaine levels were significantly higher ($p < 0.01$) in the HBC group (210.1 ± 8.7 μM) compared to the SCC group (131.1 ± 1.1 μM) and the HBA group (118.4 ± 12.8 μM). For the NC plasma samples from GD 147, the levels for choline and betaine, respectively, were 5.06 ± 0.56 μM and 147.5 ± 21.8 μM. Overall, these data suggest that the first-trimester binge alcohol treatments may reduce choline levels and metabolism but that the choline supplementation appears to increase choline levels and choline metabolism in the HBC group. In addition, choline supplementation did not influence blood alcohol concentrations (~280 mg/dL), which indicates that the fetal exposure to alcohol with the maternal intravenous alcohol infusions was not altered by choline administration.

Treatment groups

Ewes (n = 44) were randomly assigned to one of five treatments – normal control (NC) group (n = 8 ewes), saline control group (SC; isotonic saline [0.9%]) group (n = 8 ewes),

saline control plus choline (SCC; isotonic saline [0.9%] plus 10 mg/kg/day choline) group (n = 6 ewes), heavy binge alcohol (HBA; 2.5 g/kg/treatment ethanol) group (n = 14 ewes), and heavy binge alcohol plus choline (HBC; 2.5 g/kg/treatment ethanol plus 10 mg/kg/day choline) group (n = 8). The alcohol infusions in ewes modeled a weekend binge drinking pattern over the first-trimester equivalent in sheep (GD 4–41), with alcohol administered on 3 consecutive days per week, followed by 4 days without treatment (18 treatments in total).

An intravenous catheter (16 ga., 5.25-inch Angiocath™; Becton Dickinson, Sandy, UT) was placed into the jugular vein of each ewe (except for the NC group) on GD 4. Beginning on this day, alcohol (2.5 g/kg body weight) or saline was administered intravenously via a pump over a 1-h period (VetFlo® 7701B IV Vet Infusion Pump, Grady Medical, Temecula, CA). The alcohol solution was prepared by adding 95% ethanol to sterile 0.9% saline to achieve a 40% w/v alcohol solution. Solutions were prepared under aseptic conditions and passed through a 0.2-μm bacteriostatic filter. The saline control group received an infusion of isotonic saline (0.9%) that was equal in volume to the alcohol infusions. Lambs produced from ewes in the five treatment groups that entered the imaging study included 8 NC lambs (2 females, 6 males), 8 SC lambs (4 females, 4 males), 6 SCC lambs (2 females, 4 males), 14 HBA lambs (7 females, 7 males), and 8 HBC lambs (2 females, 6 males).

Ewes in the SCC and HBC treatment groups received 10 mg/kg oral choline (ReaShure® (choline chloride 28.8%; daily dose based on weight of choline, Balchem Corporation, New Hampton, NY) in which the supplement was mixed with their daily feed for the entirety of their pregnancy.

Maternal blood alcohol concentration (BAC)

To measure peak BAC, blood was drawn from the jugular vein of each ewe 1-h after alcohol infusions began on GD 6, 27, and 41, as previously described (Conover & Jones, 2012; Washburn, Sawant, Lunde, Wu, & Cudd, 2013). A 20-μL aliquot of blood was collected in a microcapillary tube and transferred into a vial containing 0.6 N perchloric acid and 4 mM n-propyl alcohol (internal standard) in distilled water. The vial was tightly capped with a septum-sealed lid and stored at room temperature until analysis by headspace gas chromatography (Varian Associates model 3900, Palo Alto, CA) within 24 h of collection.

Postnatal management

At birth, newborn lambs were weighed and measured, their navels were dipped in iodine (VetOne Stronger Iodine, 7%, MWI, Meridian, ID) and they were given oxytetracycline (Liquamycin®, LA-200®, 200 mg/mL, Pfizer Animal Health, New York, NY) 1 mL intramuscularly and selenium/vitamin E (BO-SE®, 1 mg/mL, Intervet/Schering-Plough Animal Health, Summit, NJ) 0.5 mL subcutaneously. Each ewe was checked for satisfactory milk production. Lambs were closely monitored for nursing until weaning at 2 months of age and weight gain and health status were recorded throughout the study. At 1 and 2 months of age, lambs were vaccinated using multi-species Clostridium bacterin-toxoid (Covexin 8, Intervet/Schering-Plough Animal Health, Summit, NJ) 2 mL intramuscularly. Ewes were removed from the premises after weaning and lambs were housed in outdoor covered pens. At weaning, lambs received moxidectin (Cydectin® Oral Drench for Sheep

0.1%, 1 mg/mL, Boehringer Ingelheim, St. Joseph, MO) 0.2 mL/kg body weight orally, amprolium (Corid® 9.6% Oral Solution, Merial, Duluth, GA) 0.5 mL/kg orally once daily for 10 days, and vitamin B1 (Thiamine Hydrochloride, 500 mg/mL, Rafter 8 Products, Calgary, Alberta, Canada) 1.5 mL subcutaneously once daily every third day for a total of four treatments. Weaned lambs were fed a "complete" ration (Ringmaster Start-To-Finish Show Lamb Pellets, Nutrena, Minneapolis, MN) designed to meet 100% National Research Council requirements (feed does not contain choline) as calculated by ARIES (software version 2007, University of California, Davis), and had free access to drinking water.

At 6 months of age, lambs were euthanized using sodium pentobarbital, 75 mg/kg intravenously (Beuthanasia®, Intervet/Schering-Plough Animal Health, Summit, NJ). The right and left internal carotid arteries were exposed in the cervical region. They were catheterized with tubing (2 mm, Masterflex, Cole Parmer, Niles IL) and sutured to the adjacent fascia. The corresponding right and left jugular veins were then exposed and cut transversely with a scalpel. Saline was administered intravenously through the carotid arteries to perfuse the brain over an approximate 15-min period via a peristaltic pump (Masterflex, model 7014-20, Cole Parmer, Niles, IL) until the fluid draining from the jugular veins was clear. The brain was then perfused with formalin (SP® Buffered 10% Formalin, Cardinal Health, Dublin, OH) for an additional 15-min period. The head was removed at the atlanto-occipital joint and an MRI was performed immediately.

Magnetic resonance imaging

The formalin-perfused lamb heads were scanned in a 15-channel knee coil (TxRx, Siemens Medical, USA) at the Texas A&M Institute for Preclinical Studies (TIPS) using a dedicated Siemens Magnetom Verio 3T MRI scanner (Siemens Medical, USA). Images were acquired in the sagittal plane using the following Siemens MPRAGE (Ultrafast Gradient Echo 3D) sequence: TR = 2300 ms, TE = 3.79 ms, TI = 900 ms, FA = 9, slice thickness = 0.43 mm, FoV = 256 mm × 256 mm, resolution = 0.4 mm × 0.4 mm × 0.4 mm, number of averages = 24, phase oversampling = 80%, and slice oversampling = 11.1%. Scan time was approximately 7 h per specimen with ~288 2D slices per animal (see Fig. 1).

Volumetric analysis

The whole brain volume and volumes of the cerebellum and the pituitary were manually segmented from each MRI slice (0.43-mm thickness) based on a previously described segmentation technique utilizing ITK Snap (Yushkevich et al., 2006). This software allows the lamb brains to be viewed and segmented slice-by-slice simultaneously in three dimensions. On each of the ~288 2D slices, the brain was manually segmented into the three regions of interest. ITK Snap was used to reassemble individual segmented slices into a 3D reconstruction so that the volume could be calculated (see Fig. 2). Absolute volumes were determined for the total brain, cerebellum, and pituitary gland individually. In addition, relative volumes were calculated by dividing the cerebellar and pituitary volume by the total brain volume from each subject and then multiplying by 100 to determine a percentage of the total brain (a measure of cerebellar and pituitary effects relative to effects on whole brain).

Statistical analysis

Given the unequal numbers of males and females in the NC, SCC, and HBC groups and the low number of lambs of each sex across treatment groups overall, the power to detect potential sex differences was very limited. The data were first screened for potential differences between males and females. This was to determine whether the male and female data could be combined within groups for the purposes of statistical analyses without confounding the analysis of prenatal treatment. The NC, SCC, and HBC groups each had only 2 females. The within-group ranking of the data of those subjects was determined and each fell within the range of the majority sex of the group. Therefore, the individual data did not skew the within-group distribution for any measure of the NC, SCC, or the HBC groups. For the two groups with equal numbers of males and females (SC and HBA), a two-way analysis of variance (ANOVA), with sex and treatment as the grouping factors, was calculated. None of the measures showed a significant main or interactive effect of sex, so the data of males and females were combined within groups for subsequent analyses of variance.

Statistical analysis was performed with SigmaPlot® (Version 12.5, Systat Software, Inc.). Data are presented as mean ± standard error of the mean (SEM). Treatment effects on measurements of total brain volume, cerebellum volume, pituitary volume, percentage cerebellum, and percentage pituitary were analyzed using a one-way ANOVA with a $p < 0.05$ level of significance. Body weights at birth and at 6 months and whole brain, cerebellar, and pituitary volumes were analyzed initially with a one-way ANOVA (including the normal controls). In addition, those dependent variables were also analyzed with two-way ANOVAs (involving only four groups that were instrumented and infused [alcohol or saline] as grouping factors and omitting the NC group) to assess treatment effects of alcohol and choline supplementation. Measures meeting the significance level on the univariate one-way ANOVAs were followed by pair-wise comparisons among the five treatments (four treatments with the two-way ANOVAs) within each measure, with multiple comparisons corrected by the Holm-Sidak method.

Results

Maternal blood alcohol concentration (BAC)

The mean ± SEM maternal BACs at the end of alcohol infusion (1 h; point in time at which BACs are known to peak) were 280.5 ± 12.0 mg/dL in the heavy binge alcohol (HBA) group and 277.1 ± 9.0 mg/dL in the heavy binge alcohol plus choline (HBC) group. There were no statistically significant differences in BACs between these two groups or across days of sampling.

Birth and 6-month-old body weights

Birth weights by group were 5.16 ± 0.60 kg (NC), 5.74 ± 0.33 kg (SC), 6.15 ± 0.65 kg (SCC), 4.34 ± 0.36 kg (HBA), and 4.94 ± 0.27 kg (HBC). Although these group differences were statistically significant in the overall one-way ANOVA ($F[4, 39] = 2.650$, $p = 0.048$), follow-up Holm-Sidak pair-wise group comparisons showed no significant differences among the five treatment groups, in part because the NC group was highly variable and was

intermediate between the two alcohol-exposed groups and the two saline control groups. The two-way ANOVA on birth weight, with alcohol and choline supplementation as grouping factors (omitting the NC group), yielded a significant main effect of alcohol administration, confirming the reduced birth weights of the alcohol-treated groups (F[1,31] = 8.48, p = 0.007). There were no main or interactive effects of choline supplementation on birth weight.

For body weight at 6 months, there were no significant main or interactive effects of alcohol treatment or choline supplementation. Six-month weights by group were 28.87 ± 3.74 kg (NC), 38.72 ± 4.04 kg (SC), 34.00 ± 3.07 kg (SCC), 33.22 ± 1.47 kg (HBA), and 33.75 ± 1.90 kg (HBC). The two-way ANOVA on 6-month body weights showed no significant main or interactive effects of alcohol treatment or choline supplementation.

Whole brain volume

As shown in Fig. 3 (top row), significant effects among the five treatment groups were evident in the whole-brain volume measures (one-way ANOVA, F[4, 39] = 9.575, p < 0.001). Follow-up pairwise comparisons (Holm-Sidak corrected) confirmed that the whole brain volumes of the two alcohol-exposed groups (HBA, HBC) were significantly reduced relative to the three control groups (NC, SC, SCC). There were no significant differences between the heavy binge alcohol groups, with (HBC) and without (HBA) choline supplementation, or among the three control (NC, SC, SCC) groups. In addition, the two-way ANOVA with alcohol and choline supplementation as grouping factors (omitting the NC group) yielded a significant main effect of alcohol administration, confirming the reduced whole-brain volume of the alcohol-treated groups (F[1,32] = 35.67, p < 0.001). There were no main or interactive effects of choline supplementation on whole-brain volume.

Cerebellar and pituitary volumes

In one-way ANOVAs for absolute volumes of the cerebellum and pituitary, there were no significant differences among the five treatment groups in either the cerebellum (F[4,39] = 2.280, p = 0.078) or pituitary (F[4,39] = 0.774, p = 0.549) (see Fig. 3; top row). There were also no significant differences in the relative volume, expressed as percent of the total brain, for the cerebellum (F[4,39] = 0.959, p = 0.441) and pituitary (F[4,39] = 1.104, p = 0.368) (see Fig. 3; bottom row). However, with a two-way ANOVA for cerebellar volume involving the four prenatally infused groups (alcohol or saline, omitting the NC group), the main effect of alcohol administration reached statistical significance, confirming that alcohol reduced cerebellar volumes in the HBA and HBC groups (F[1,32] = 9.29, p = 0.005). There were no main or interactive effects of choline supplementation on the cerebellar volume. For pituitary volume, the two-way ANOVA yielded no significant main or interactive effects of alcohol treatment or choline supplementation.

Discussion

The use of choline as an interventional strategy to lessen the effects of prenatal alcohol exposure on the developing fetus has been studied prenatally and postnatally in the rodent,

showing some beneficial improvements in memory, behavior, and cognition (Monk et al., 2012; Thomas et al., 2004, 2009, 2010; Wagner & Hunt, 2006). Currently, there is one published randomized clinical trial (from the CIFASD initiative) involving prenatal choline supplementation as a component of a multivitamin and mineral (MVM) supplementation trial (Coles et al., 2015). That study indicated benefits of the MVM supplements in infants on Bayley Mental Development scores (particularly in males), but there were no significant additional benefits of choline beyond these scores. In addition, the MVM supplements did not prevent lower birthweights and smaller head circumferences in the prenatally alcohol-exposed infants. The current findings in this sheep model suggest that the reduced brain size and lower birth weights associated with first-trimester binge alcohol exposure are not likely ameliorated with the dose of choline used (matched between the sheep study and the clinical trial), even if the choline supplements were started early in pregnancy and continued until birth.

Choline doses used in this study (10 mg/kg per day) were carefully matched to the dose used in the CIFASD clinical trial. However, on a mg/kg basis, this dose is more than an order of magnitude lower than the doses used in rodent studies that showed benefits in neurobehavioral and physical developmental outcomes (typically ~250 mg/kg per day). This raises the appropriate question as to whether the dose of choline needed to produce beneficial effects needs to be much higher than is currently used for clinical interventions. The failure of maternal choline supplementation to mitigate brain volume reductions associated with prenatal alcohol exposure suggests that any potential cognitive, cellular, and other benefits of choline in the sheep model of FASD, prenatally or postnatally, remain to be determined. Other complex questions about pharmacokinetics, metabolism, and bioavailability of choline (as a nutraceutical) across species (rodents, sheep, and humans) also need to be addressed in future studies. Additionally, most women are likely to consume varying amounts of choline in their daily diet. In the Ukraine clinical trial, the supplement they received was in addition to whatever amount of choline was present in their daily diet (Coles et al., 2015). Since the choline requirements in ruminants are not well established, most commercial rations do not contain choline. Therefore, we can only say with certainty that the ewes received the 10 mg/kg/day choline for the duration of their pregnancy. If doses of choline (as a nutraceutical, rather than just to correct a deficiency) need to be high (as used in the rodent studies), this may limit the feasibility in clinical settings due to tolerability. Currently, there is one study in children (aged 2–5 years old) with FASD that assessed tolerability and potential adverse effects associated with choline supplementation (Wozniak et al., 2013), but this information is not known for pregnant women.

To our knowledge, there are no published human or animal model studies that evaluate volumetric structural brain measures with choline supplementation given either prenatally or postnatally with heavy binge-like alcohol exposure. The current study of lambs born to ewes given alcohol during the first-trimester equivalent of gestation was intended to assess quantitative measures of three easily obtained structural volumes known to be affected by prenatal alcohol exposure in peripubertal lambs (modeling early adolescent children), and to determine whether prenatal choline supplementation has measurable effects on brain volume.

This is the first report of quantitative MRI showing reduced total brain volume in 6-month-old lambs modeling heavy weekend binge drinking over the first trimester. Our finding of total brain volume reductions in alcohol-exposed lambs are consistent with numerous neuroimaging studies in children and young adults with known prenatal alcohol exposure and FASD, regardless of whether they meet the full diagnostic criteria for FAS (Moore et al., 2014). Brain imaging studies in murine models have also shown whole-brain volume reductions (O'Leary-Moore et al., 2010; Parnell et al., 2009, 2013). Importantly, the prenatal alcohol-induced reductions in brain volumes observed in this sheep model occurred with a weekend binge exposure model that produced BAC profiles consistent with binge drinking (~280 mg/dL).

Significant reductions in absolute cerebellum volumes reported in this study (compared to saline-infused controls) were consistent with neuroimaging findings in humans (Archibald et al., 2001; de Zeeuw et al., 2012; O'Hare et al., 2005; Riikonen et al., 1999), while a contrasting study in rats found that cerebellar weight, volume, Purkinje cell number, and granule cell density were only reduced in the third trimester and all three trimester exposure models. Moreover, data from the first-trimester exposure did not differ from the control groups (Maier, Miller, Blackwell, & West, 1999). It is reasonable to conclude that cerebellar volume reduction is more severe when alcohol exposure extends beyond the first trimester, but even first-trimester exposure can lead to significant cerebellar deficits. There would be value in analyzing regional volumes of other cortical and subcortical brain structures to determine whether they are more susceptible to first-trimester alcohol exposure.

Consistent with prior murine studies (Godin et al., 2010; O'Leary-Moore et al., 2010; Parnell et al., 2009, 2013, 2014), neither absolute nor relative pituitary volume differed significantly among the five treatment groups. However, two studies in GD 17 mice showed an increased pituitary volume following exposure to alcohol during GD 8 and GD 12–16 (Parnell et al., 2009, 2014).

Limitations with this model include increased time, effort, and expense associated with accruing representative numbers of sheep for evaluation. In addition, because of the high expense of anesthesia and long MRI scan times to acquire high-resolution images, animals were euthanized and their brains were perfused with formalin immediately prior to scanning. Although possible, we do not believe that fixation would differentially affect alcohol-exposed brains versus normal control brains.

In conclusion, our findings confirm that heavy binge alcohol exposure in the first trimester in the sheep model produces significant brain volume reductions in peripubertal lambs and that maternal choline supplementation at 10 mg/kg/day failed to prevent these reductions.

MRI-based volumetric findings in the brain are comparable to outcomes reported for children and adolescents with FASD, further supporting the translational advantages of the sheep model. The lack of brain volume protection by gestational choline treatments suggest that even though choline supplementation in preclinical rodent studies benefits neurodevelopmental and cognitive functions, it may not prevent reduction in brain volume.

Future studies in this sheep model will evaluate behavioral and additional brain structure volumetrics.

Acknowledgments

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Highlights

- MRI-derived brain volumetrics as a diagnostic tool for FASD in sheep model

- Prenatal binge alcohol results in decreased brain volume in peripubertal sheep.

- Maternal choline supplementation does not alter brain volume in binge-exposed lambs.
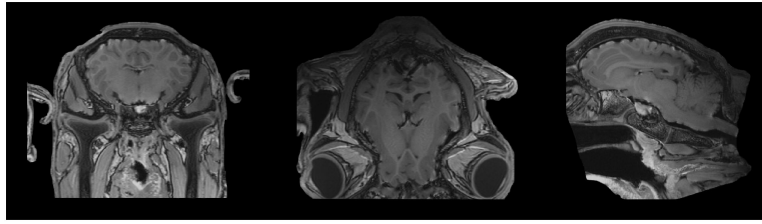
Fig. 1.
Magnetic resonance imaging- (MRI) derived images of a 6-month-old lamb brain. From left to right, representative transverse (or coronal/frontal in humans), dorsal (or horizontal/transverse/axial in humans), and sagittal views show high resolution.
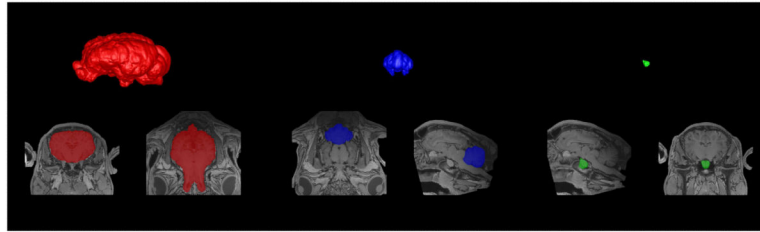
Fig. 2.
Manually segmented MRI-derived images of a normal 6-month-old lamb brain. Scans are color-coded and reconstructed in 3D in ITK Snap (Yushkevich et al., 2006), by which volumes are calculated: whole brain (red; transverse [coronal/frontal in humans] and dorsal [horizontal/transverse/axial in humans] views shown), cerebellum (blue; dorsal [horizontal/transverse/axial in humans] and sagittal views shown), and pituitary (green; sagittal and transverse [coronal/frontal in humans] views shown).
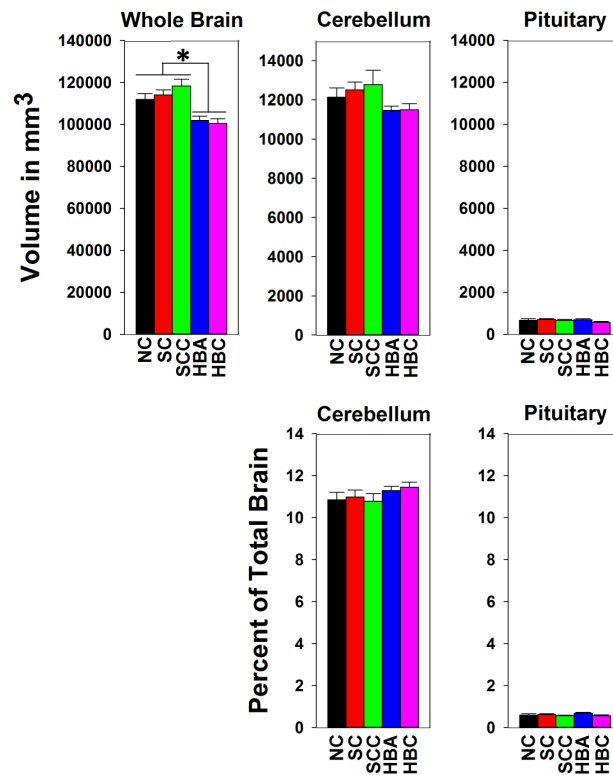
Fig. 3.
Treatment effects on the whole brain, cerebellum, and pituitary volumes (top row) and treatment effects on the cerebellum and pituitary as a percentage of total brain size (bottom row). The y-axis (top row) indicates the mean volume ($\pm$ SEM) in mm^3 for whole-brain volume, cerebellum, and pituitary. Note the scale for the whole brain in the top left panel (maximum of 140,000 mm^3) differs from that of the cerebellum and pituitary in the two top right panels (maximum of 14,000 mm^3). The bottom panel is the percent total brain (maximum 14%). The ANOVA α level was 0.05, followed by the Holm-Sidak method for pair-wise comparisons ($p < 0.05$). Significant group differences are indicated within each panel. An asterisk (*) indicates that the volumes of the three control groups (NC, SC, SCC) were significantly greater than the two alcohol-exposed groups (HBA and HBC); NC, SC, and SCC did not differ from each other, and HBA and HBC did not differ from each other.