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Effects of all three trimester moderate binge alcohol exposure on the foetal hippocampal formation and olfactory bulb

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Abstract

Objective—Pre-natal alcohol exposure results in injury to the hippocampus and olfactory bulb, but currently there is no consensus on the critical window of vulnerability. This study tested the hypothesis that pre-natal exposure to a moderate dose of alcohol during all three trimester-equivalents alters development of the hippocampal formation and olfactory bulb in an ovine model, where all brain development occurs pre-natally as it does in humans.

Research design and methods—Pregnant sheep were divided into saline control and a binge drinking groups (alcohol dose 1.75 g kg⁻¹; mean peak blood alcohol concentration 189 + 19mg dl⁻¹).

Outcome and results—The density, volume and total cell number were not different between groups for the dentate gyrus, pyramidal cells in the CA1 and CA2/3 fields and mitral cells in the olfactory bulb.

Conclusions—A moderate dose of alcohol administered in a binge pattern throughout gestation does not alter cell numbers in the hippocampus or olfactory bulb and exposure during the third trimester-equivalent is required for hippocampal injury, unless very high doses of alcohol are administered. This has important implications in establishing the sensitivity of imaging modalities such as MRI in which volumetric measures are being studied as biomarkers for pre-natal alcohol exposure.

Keywords

Ethanol; foetal alcohol syndrome; hippocampus; olfactory bulb; sheep

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Declaration of interest

The authors report no conflicts of interest.

Introduction

Maternal alcohol exposure can lead to debilitating outcomes in the offspring that range from mild behavioural and learning disabilities to severe physical and mental deficits that are collectively termed Foetal Alcohol Spectrum Disorders (FASD) [1, 2]. Serious behavioural problems and altered performance in learning and/or memory tasks in children with FASD [3] are thought to be caused by hippocampal injury [4]. Further support for this conclusion is provided by reported deficits in place learning and spared cue-navigation among children with FASD [5] and by magnetic resonance images displaying morphologic left–right asymmetries in the hippocampus of children with FASD [6].

There are no quantitative studies of hippocampal neuron numbers in children pre-natally exposed to alcohol. However, there are a number of animal model studies that have addressed the issue of when, relative to development, the hippocampus is vulnerable to pre-natal alcohol exposure. Differences in the number of hippocampal neurons in alcohol-exposed subjects have been reported in these studies and the effect on neuron number varies depending on the blood alcohol concentration (BAC), region of hippocampal formation and time period of alcohol exposure. It has been demonstrated in the rat model that the hippocampus is vulnerable to pre-natal alcohol exposure and that the third trimester exposure is required for injury; in addition, during the third trimester equivalent exposure in the rat, alcohol has been preferentially harmful to different cell types within the foetal hippocampal formation and these effects are different depending on the BAC [7–10]. It is important to note that the third trimester-equivalent of human brain development in the rat occurs post-natally, thus experiments are being conducted on neonates rather than foetuses; in the sheep model, the third trimester-equivalent of human brain development occurs *in utero*, thus more closely resembling what happens in humans and allowing for effects of placental and maternal–foetal interactions [11, 12]. Since the third trimester-equivalent has been established as a critical period of hippocampus vulnerability in the rat, investigating this time period in a more translational model like sheep will allow for more accurate extrapolation to humans. Further, the unbiased stereological and optical disector methods used in this study provide more accurate cell counts than methods used in other studies (such as density measures only or stereology without the optical disector method).

While the hippocampus has long been known to be important in spatial learning and memory, recent research has focused on the contributions of extrahippocampal regions to spatial learning and memory. Lesions of the entorhinal cortex and olfactory bulb lead to deficits in spatial working memory and reversal learning. The piriform, perirhinal and lateral entorhinal cortical areas receive common excitatory inputs from the olfactory bulb and are inter-connected to form a hippocampal circuit implicated in modulating memory [13]. The olfactory bulb undergoes neurogenesis at the same time as the cerebellum and it has been well established that the cerebellum is sensitive to pre-natal alcohol exposure [14–16]. A permanent deficit in the number of mitral cells in the olfactory bulb has been demonstrated in the rat model as a result of alcohol exposure during the third trimester equivalent [17–19] and the first two trimester equivalents [20]. In addition, Peiffer et al. [21] observed olfactory bulb agenesis and morphological defects of the olfactory stalks in their report on human

alcohol teratology. Early olfactory learning in rodents is also used as a model to study neurobehavioural deficits due to pre-natal alcohol exposure [22]. Considering the importance of the olfactory bulb in various learning and memory tasks, it renders this unique brain region a relevant target for investigation.

The purpose of this study was to test the hypothesis that pre-natal exposure to a moderate dose (1.75 g kg^{-1}) of alcohol during all three trimester-equivalents alters development of the hippocampal formation and the olfactory bulb in an ovine model, where all three trimester-equivalents of human brain development occur pre-natally as they do in humans. The pyramidal cells of the CA1 and CA 2/3 sub-fields, the granule cells of the dentate gyrus and the mitral cells of the olfactory bulb were the specific regions examined. A weekend binge drinking pattern, common in women who abuse alcohol during pregnancy, was selected for the alcohol administration paradigm [23–26]. Since it has been reported that all three trimester alcohol exposure at a dose of 1.75 g kg^{-1} resulted in cerebellar neuronal loss in foetal sheep, this same dose was chosen to study hippocampal and olfactory neuronal deficits in this study [27]. The moderate blood alcohol concentration (BAC) produced by the alcohol dose in this study will provide further insight with respect to BAC-dependent alcohol effects in the hippocampus.

Materials and methods

Animals and breeding

This study utilized the fetuses of 12 adult pregnant ewes. The experimental procedures were approved by the Institutional Animal Care and Use Committee at Texas A&M University. Suffolk ewes (aged 2–5 years) maintained on coastal Bermuda grass pasture and supplemented with alfalfa hay were bred under controlled conditions. Time dated pregnancies were achieved by controlling the oestrous cycle through the use of progesterone impregnated vaginal implants (EAZI-BREED™, CIDR®, Pharmacia & Upjohn Ltd., Auckland New Zealand). Implants were removed 11 days after placement, at which time prostaglandin F2 α (LUTALYSE®, Pharmacia & Upjohn, Kalamazoo, MI, 20 mg) was administered intramuscularly. 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 25, 60 and 90 days later to confirm pregnancy. On gestational day (GD) 4, all subjects were moved into individual pens, but were able to see herd mates in the adjacent pens at all times. Conditions of constant temperature (22 °C) and fixed light dark cycle (12:12) were maintained. Once confined, both the saline and alcohol treatment group subjects received 2 kg day^{-1} of a complete ration (Sheep and Goat Pellet, Producers Cooperative Association, Bryan, TX) and all subjects in both groups consumed all feed offered.

Alcohol dosing protocol

Pregnant ewes were divided into two treatment groups, an alcohol group (alcohol dosage of 1.75 g kg^{-1} body weight) and a saline control group that received 0.9% saline of a volume and at an infusion rate equivalent to that of the alcohol dose. Infusion solutions were delivered intravenously by peristaltic pump (Masterflex, model 7014-20, Cole parmer, Niles, IL) through a $0.2 \mu\text{m}$ bacteriostatic filter; pumps were calibrated before infusion.

Alcohol infusions were 40% w/v in sterile saline and administered over 1 hour. Alcohol was administered on 3 consecutive days followed by 4 days without alcohol to mimic a binge drinking pattern (common in women who abuse alcohol during pregnancy) beginning on GD 4 and continuing until GD 132 (which corresponds with the end of the third trimester of human brain development, the peak of the brain growth spurt in sheep) [11,12]. This resulted in a total of 57 infusions over the course of 19 weeks.

Experiment protocol

The experiments began on GD4 and were terminated on GD 133 and have been described previously [27,28]. In brief, on GD 4, an intravenous catheter (16 gauge, 5.25 in Angiocath™ Becton Dickinson, Sandy, UT) was placed percutaneously into the jugular vein of the ewe. On the day of infusions, ewes were connected to the infusion pump by 8:30 am and alcohol (or saline) was infused continuously over 1 hour. On GD 42, after pregnancy was confirmed ultrasonographically, the ewes underwent surgery to implant femoral arterial and venous vascular access ports (V-A-PTM, Model CP 47 P, Access Technologies, Skokie, IL). The ewes were not surgically instrumented until after the first trimester to avoid early embryonic losses. For the remainder of the experiment, infusions were then given through the venous port and blood was sampled from the arterial port.

Blood alcohol concentration (BAC) measurement

Blood was drawn from the jugular vein catheter on GD 6 and GD 40 and from the femoral artery port on GD 90 and GD 132; samples were collected 1 hour following the commencement of alcohol infusions for the measurement of BAC. A 20µl 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 [29], with the exception of the column (DB-wax, Megabore, J&W Scientific Folsom, CA) and the carrier gas (helium) used [30].

Foetal hippocampal and olfactory bulb tissue processing

On GD 133, the ewes were euthanized using sodium pentobarbital (75 mg kg⁻¹, intravenously) and the foetuses were removed from the uterus. The foetal brains were perfused with saline followed by cold fixative solution containing 1.25% paraformaldehyde and 3% glutaraldehyde in phosphate buffer (pH 7.4). The brains were removed from the skull and stored in additional fixative until processed for stereological cell counting. The foetal brain was divided parasagittally and the right olfactory bulb and hippocampus were removed. 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; Historesin™ Embedding kit, Leica, Wetzlar, Germany). Following infiltration, the tissue was embedded in a solution containing 1ml dimethyl sulphoxide (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 mounted serially on

glass slides, stained with cresyl violet and coverslipped. A representative coronal section from a normal control hippocampal formation is presented in Figure 1.

Stereological cell counting

The total number of foetal hippocampal pyramidal cells in the CA1 and CA2/3 fields, granule cells in the dentate gyrus and mitral cells in the olfactory bulb were estimated using unbiased stereological cell counting techniques as described in previous publications [15,30,31]. The term CA2/3 refers to the summed CA2 and CA3 fields, which were combined due to the difficulty in differentiating these two fields accurately at this developmental stage. 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 colour video camera (model 2040, Jai, Copenhagen, Denmark). The reference volume was estimated using the Cavalieri's Principle and was calculated by the equation $V_{ref} = \sum \pi_i \times A(\pi_i) \times t$, where $\sum \pi_i$ is the total number of points (π_i) counted, $A(\pi_i)$ is the known area associated with each point and t is the known distance between two serial sections counted. The GRID[®] software provided templates of points in various arrays that were used in point counting for reference volume estimation. The cell density was determined by following the optical disector method, which was calculated using the formula $N_v = \sum Q / (\sum disector \times A(fr) \times h)$, where $\sum Q$ is the sum of the hippocampal/olfactory cells counted from each disector frame, $\sum disector$ is the sum of the number of disector frames counted, $A(fr)$ is the known area associated with each disector frame and h is the known distance between two disector planes. The placement of the disector frames was determined by the GRID[®] software in a random manner. The estimated total number of cells in the olfactory bulb, dentate gyrus, CA1 and CA2/3 fields were then calculated by multiplying the reference volume of the respective regions and the numerical density of cells within this reference volume as described before [15,30,31].

Data analysis

Data are presented as mean ± SEM. All stereology measures were analysed using unpaired two-tailed *t*-tests to compare between the control and alcohol groups. Significance was determined *a priori* at $p < 0.05$.

Results

There were no differences in foetal body or brain weights between groups. The mean BACs measured on GD 6, 40, 90 and 132 in the alcohol group peaked at 1 hour (which coincided with the end of infusion) and did not differ significantly between days, so were therefore combined. The mean peak BAC was 189 ± 19 mg dl⁻¹. Subjects remained conscious throughout and after the alcohol infusion, but appeared ataxic if encouraged to walk shortly after the end of the infusion. As reported previously, maternal hypercapnea, acidemia and normoxemia was present with every bout of alcohol [27,32,33].

Stereology data

In response to maternal alcohol binging throughout gestation, no differences in the total number of foetal dentate gyrus granule cells, CA1 pyramidal cells, CA2/3 pyramidal cells or mitral cells of the olfactory bulb were found between groups (Table I).

Although it did not reach significance, the density of dentate gyrus granule cells trended higher by 36% in alcohol exposed fetuses compared to the saline control group. No differences in the density of CA1 pyramidal cells, CA2/3 pyramidal cells or mitral cells of the olfactory bulb were found between groups (Table II).

Although it did not reach significance, the volume of the dentate gyrus granule trended lower by 16% in alcohol exposed fetuses compared to the saline control group. No differences in the reference volume of CA1 pyramidal cells, CA2/3 pyramidal cells or mitral cells of the olfactory bulb were found between groups (Table III).

Discussion

The effects of all three trimester-equivalent alcohol exposures on the ovine foetal hippocampal formation

A moderate dose of alcohol (creating BACs of 189 mg dl⁻¹) administered in a 3 consecutive day per week binge pattern throughout gestation did not change the total dentate gyrus cell number, but the density exhibited a trend increase of 36% ($p = 0.0773$) and the volume exhibited a trend decrease of 16% ($p = 0.0883$), suggesting that development could be altered. Density, reference volume and the number of pyramidal cells in the CA1 and CA2/CA3 fields were not altered by this pre-natal alcohol exposure paradigm. This potentially selective effect of pre-natal alcohol exposure on the dentate gyrus is in agreement with studies performed in the rat model. West et al. [7] demonstrated that third trimester-equivalent exposure using a higher dose of alcohol (BAC 380 mg dl⁻¹) produced a 10% increase in the density of granule cells in the dentate gyrus, but no change in the density of CA1 or CA3 pyramidal cells. Miller [8] observed an increase in dentate gyrus granule cell number in post-natal rat pups (third trimester-equivalent exposure) in response to moderate alcohol exposure (BAC, 132 mg dl⁻¹) and a decrease in their number in response to BACs of 339 mg dl⁻¹. In this same study, no decrease in CA1 pyramidal cell number was observed following third trimester-equivalent exposure unless the BAC exceeded 300 mg dl⁻¹. Similarly, Livy et al. [9] demonstrated a decrease in dentate gyrus granule cells, as well as a decrease in CA1 and CA3 pyramidal cell numbers in response to high doses of alcohol (BAC's 268–359 mg dl⁻¹), but only when the exposure period included the third trimester-equivalent of human brain development. Bonthius and West [34] also demonstrated decreased CA1 pyramidal cells in response to post-natal alcohol exposure at a higher dose (BAC 361 mg dl⁻¹), while no such reduction was found in response to a BAC of 190 mg dl⁻¹. Maier and West [10] found that none of the hippocampal cell types were altered when alcohol exposure was limited to the first two trimester-equivalents (BAC's 136–422 mg dl⁻¹).

This study and those conducted in the rodent substantiate the conclusion that all three trimester-equivalent exposures to moderate doses of alcohol do not alter the number of

pyramidal cells in the developing hippocampus, whereas exceptionally high BACs may selectively reduce CA1 pyramidal cell number. Further, there is a preferentially selective and dose-dependent effect on the foetal dentate gyrus in response to pre-natal alcohol exposure, with changes exhibited in the density and/or number of dentate gyrus granule cells and with none of these alterations being observed when the exposure period does not include the third trimester-equivalent. In addition, rodent studies using other modalities, such as magnetic resonance imaging and behavioural testing, to evaluate the hippocampus have also found that change in volume may not be a sensitive indicator of pre-natal alcohol exposure, but that functional deficits and surface and shape changes are present that indicate damage does indeed occur [35–37]. The findings from this study utilizing unbiased stereologic analysis highlight the need to critically evaluate the sensitivity as well as specificity of screening methods being developed to assess injury from prenatal alcohol exposure.

Interestingly, it has previously been reported that this same pre-natal alcohol exposure paradigm in sheep (all three trimester-equivalent alcohol exposures, BAC 189 mg dl⁻¹) reduced the estimated number of cerebellar Purkinje cells [27], a finding which, when taken together with this report, further demonstrates the regionally selective actions of alcohol on the foetal brain. Understanding how different brain regions and cell types are differentially vulnerable to cell loss from pre-natal alcohol exposure may provide clues to the mechanisms underlying the cell loss (for example, cell death or reduced proliferation).

The effects of all three trimester-equivalent alcohol exposures on the ovine foetal olfactory bulb

Similar to the findings from the evaluation of hippocampal pyramidal cells, there was no change in the number, density or volume of mitral cells in the foetal sheep olfactory bulb in response to this moderate binge alcohol exposure paradigm throughout gestation. Findings derived using a rat model and a third trimester-equivalent only alcohol exposure paradigm demonstrated a reduction in mitral cell number and reference volume, but no change in cell density in response to alcohol treatment (BAC's ranged from ~209–329 mg dl⁻¹) [14]. In this same study, there were no changes in mitral cell numbers if the alcohol was administered throughout all three trimester equivalents, which is consistent with findings in an all three trimester exposure in the sheep. The lack of change in mitral cell number does not exclude the possible presence of functional abnormalities, thus the possibility of functional but not structural injury in response to pre-natal alcohol exposure should be explored further.

Conclusion

It has been demonstrated that a moderate dose of alcohol administered throughout gestation does not alter the total number, density or volume of CA1 and CA2/3 pyramidal cell in the ovine foetal hippocampus nor the total number, density and volume of mitral cells in the olfactory bulb. The dentate gyrus of the foetal hippocampal formation is more vulnerable to damage from consumption of a moderate dose of alcohol *in utero* throughout gestation compared to CA1 and CA2/3 pyramidal cells and mitral cells in the olfactory bulb and the changes are dose-dependent [8]. The trend changes observed in the foetal dentate gyrus cell

density and volume (Tables II and III) suggest that the BAC and binge pattern of alcohol used in this study may represent a threshold for significant alterations. This has important implications in establishing the sensitivity of imaging modalities such as MRI in which volumetric measures are being studied as biomarkers to distinguish alcohol exposed from normal controls [38]. These findings are also significant because children with FASD exhibit behavioural deficits that include altered performance in tasks that involve hippocampal function, such as learning and memory [3–5] and these impairments have been correlated to hippocampal structural deficits [6]. The lack of change in cell number does not exclude the possible presence of functional abnormalities or other non-structural changes such as alterations in neurotransmitter or neurotrophic factors [39]. Further exploration of the possibility of functional, but not structural, injury in response to pre-natal alcohol exposure is warranted. Besides illustrating the regional vulnerability of the brain to pre-natal alcohol exposure, the findings from this study further establish (using an animal model that closely models human development and a pre-natal third trimester-equivalent alcohol exposure) that the hippocampus is an important target of pre-natal alcohol exposure and provides support that the third trimester-equivalent of human brain development exposure is required for injury.

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Figure 1.
Representative coronal section of the ovine foetal hippocampal formation from the control group. The section illustrates well-formed dentate gyrus, CA1 and CA2/3 pyramidal fields by gestational day 133 (term=147).

Table 1

Mean total cell number $\times 10^6$ for each region/cell type counted in both groups.

Region/cell type	Total cell number					
	Saline			Alcohol		
	Mean	SEM		Mean	SEM	p value
DG granule cells	2.586 482 8	0.211 262 3		2.551 656 4	0.167 398 8	0.8998
CA2/3 pyramidal cells	1.0409 888	0.080 634 1		1.035 334 8	0.137 837 3	0.9725
CA1 pyramidal cells	0.612 899 3	0.014 240 96		0.785 433 2	0.152 174 7	0.4271
Olfactory mitral cells	258 619.5	0.013 507 4		217 353.1	0.022 767 3	0.1501

n = 9 per group for all regions/cell types.

Table II

Mean density in (cells mm⁻³) for each region/cell type counted in both groups.

Region/cell type	Density				p value
	Saline		Alcohol		
	Mean	SEM	Mean	SEM	
DG granule cells	0.901	0.029 4	1.407	0.067 9	0.0773
CA2/3 pyramidal cells	0.163	0.009 95	0.249	0.053 94	0.1488
CA1 pyramidal cells	0.213	0.038 5	0.283	0.030 1	0.1804
Olfactory mitral cells	0.0652	0.006 78	0.0533	0.004 27	0.9107

n = 6 per group for all regions/cell types.

Table III

Mean volume in $\text{mm}^3 \times 10^6$ for each region/cell type counted in both groups.

Region/cell type	Volume					
	Saline			Alcohol		
	Mean	SEM		Mean	SEM	<i>p</i> value
DG granule cells	2854.276 823 2	151.698 452 6		2456.894 240 2	145.889 643 2	0.0883
CA2/3 pyramidal cells	6377.226 262 3	299.994 885 0		4974.924 262 3	997.123 714 5	0.2078
CA1 pyramidal cells	2735.826 245 5	286.568 883 5		2682.332 436	303.685 497 4	0.9006
Olfactory mitral cells	4237.976 971 5	548.268 033 8		4337.351 070 3	667.508 628 4	0.1656

n = 6 per group for all regions/cell types.