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The Effects of Binge Alcohol Exposure in the 2nd Trimester on the Estimated Density of Cerebral Microvessels in Near-Term Fetal Sheep

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Abstract

Heavy fetal alcohol exposure is associated with a spectrum of neurological abnormalities, although the mechanism of injury is largely unknown. We previously reported attenuated cerebral blood flow response to hypoxia in fetal and newborn sheep which were exposed to alcohol earlier in pregnancy. One possible mechanism for this effect of alcohol on the developing cerebral vasculature is a decrease in cerebral microvessel density, similar to its effect on developing neurons. Therefore, we tested the hypothesis that prenatal alcohol exposure decreases cerebral microvessel density. Pregnant ewes received intravenous infusions of ethanol or saline during days 60–84 of gestation (term=150 days) and at 125 days of gestation we obtained the fetal brains for study. We immunohistochemically labeled vessels of the left cerebral forebrain hemispheres with antibody to endothelial nitric oxide synthase and then obtained unbiased stereological estimates of cerebral microvessel density using a modified optical fractionator method. We studied 20 fetal brains of which 9 were alcohol-exposed, 11 were saline-controls, and all were products of a twin gestation. Although brain and body weights were not different between groups, the alcohol-exposed group had significantly lower brain weight as a percentage of body weight. Estimates of cerebral microvessel density were not significantly different between alcohol-exposed and saline-control groups: 12.7 ± 8.7 and 9.1 ± 2.8 microvessels per mm^3 , respectively (mean $\pm$ SD, $p=0.32$). Since there is no change in estimated cerebral microvessel density after prenatal alcohol exposure, we conclude that decreased cerebral microvessel density is not a likely explanation for attenuated cerebral blood flow in response to hypoxia.

Keywords

Fetal Alcohol Syndrome; Cerebral Microvessel Density; Sheep

1. Introduction

Fetal alcohol syndrome (FAS) is a constellation of physical, behavioral, and cognitive abnormalities first described by Jones and Smith in 1973 (Jones et al., 1973). Central nervous

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system (CNS) abnormalities after fetal alcohol exposure can include microcephaly (Rosett et al., 1983), partial to complete agenesis of the corpus callosum (Sowell et al., 2001), and neuronal loss (Nowoslawski et al., 2005). Cognitive abnormalities include mental retardation, learning disabilities, and behavioral abnormalities that persist into adulthood (American Academy of Pediatrics, 2000; Streissguth et al., 1991). Proposed mechanisms for these alcohol-related CNS and cognitive abnormalities include direct or indirect neurotoxic effects (West et al., 1994), inflammatory injuries (Goodlett et al., 1993), poor intrauterine nutrition (Morgane et al., 1993), and poor placental function (Gordon et al., 1985). In addition, we and others are investigating possible cerebrovascular effects of prenatal alcohol exposure. We previously reported significantly attenuated cerebral blood flow responses to hypoxia in fetal (Mayock et al., 2007) and newborn (Gleason et al., 1997) sheep which were exposed to alcohol early in gestation. Kelly et al. have demonstrated regional alterations in the development of the cerebral microvasculature in rats after postnatal alcohol exposure (Kelly et al., 1990). Prenatal alcohol exposure in rats causes decreased generation and impaired migration of cortical neurons as well as “simple” dendritic arborization (Detering et al., 1981; Hammer and Scheibel, 1981; Miller, 1986). We hypothesized that alcohol may similarly decrease cerebral microvessel density in the developing brain.

We used a maternal binge model of second trimester fetal alcohol exposure in sheep to test the hypothesis that prenatal alcohol exposure causes decreased fetal cerebral microvessel density. We labeled the vascular channels immunohistochemically with antibody to endothelial nitric oxide synthase (eNOS), present in endothelial cells lining vessel walls, and quantitated microvessels using unbiased stereology. Understanding potential mechanisms for alcohol's effect on the developing cerebral circulation is important for the development of preventive and treatment strategies for FAS.

2. Results

2.1. Animals, Maternal and Fetal Measurements

We studied 20 fetal brains which were the progeny of 15 ewes. Nine brains were alcohol-exposed (2 male, 7 female) and eleven were saline-controls (6 male, 5 female). All fetuses were products of a twin gestation. Two of the alcohol-exposed group and 8 of the saline-control group were twin pairs. Seven of the alcohol-exposed group and 3 of the saline-control group fetuses were the only twin of their pair in the study. There were no significant differences between the groups in brain weight and body weight. However, the alcohol-exposed group had a significantly lower brain weight as a percentage of body weight (Table 1). Average maternal blood alcohol concentration immediately after the infusion was 215.6 ± 20 mg/dL. For comparison, the legal limit for driving while intoxicated in most states is 80 mg/dL.

2.2. Immunohistochemistry

Microvessel walls were densely stained brown with DAB eNOS immunoreaction product. Morphology of vascular channels was characterized microscopically by birefringence around all vessels and an unmistakable lumen, whether a vessel was viewed in cross-section, diagonal, or longitudinal section. Morphology combined with eNOS immunoreactivity allowed us to unambiguously identify vessels upon microscopic examination and revealed that all vascular structures were densely labeled (Fig. 1).

2.3. Stereology

We observed no gross qualitative differences in the distribution or apparent number of microvessels. This was confirmed by our quantitative analysis in which the estimated fetal cerebral microvessel density was 12.7 ± 8.7 and 9.1 ± 2.8 microvessels per mm^3 in the alcohol-exposed and saline-control fetuses, respectively (mean $\pm$ SD, $p=0.32$). (Fig. 2)

3. Discussion

The major finding of this study is that prenatal alcohol exposure during the 2nd trimester, in a manner that mimics repeated maternal alcoholic binge exposure, does not alter the estimated density of cerebral microvessels in near-term fetal sheep. We chose our 2nd trimester binge alcohol model for several reasons. First, binge drinking is more clinically relevant than continuous alcohol exposure which is commonly used in rodent models. Second, the 2nd trimester may be a particularly vulnerable time for brain injury. For example, neuronal loss in rats is reportedly the greatest with fetal alcohol exposure during the 2nd trimester (Miller and Potempa, 1990). In addition, the brain growth spurt in sheep also occurs in the second trimester (Dobbing and Sands, 1979). Finally, we have demonstrated attenuated cerebral blood flow in response to hypoxia after 2nd trimester alcohol exposure (Mayock et al., 2007). However, our findings do not indicate that decreased microvessel density played a role in this attenuated cerebral blood flow in response to hypoxia but this lack of effect of fetal alcohol exposure on microvessel density could be consistent with lack of effect on cerebrovascular reactivity to hypercapnia (Nowoslawski et al., 2005).

Our findings are of interest in relation to those of Kelly et al. whose rat model of neonatal alcohol exposure demonstrated regional alterations in the development of the cerebral microvasculature which included no change in capillary density in the cerebellum, decreased capillary density (but not capillary diameter) in the dentate gyrus of the hippocampus, and no overall change in capillary density (but increased capillary diameter) in the hippocampus (Kelly et al., 1990). There are important methodological differences between our study and Kelly et al. First, we used a pattern of prenatal alcohol exposure that modeled a human pattern of binge drinking. Binges produce a high blood alcohol concentration (BAC) which has been shown to be a key risk factor in determining severity of brain damage (Pierce and West, 1986). Kelly et al. also used a binge model but alcohol was fed to neonatal rat pups via a gastrostomy tube over a two day postnatal period compared to our fetal exposure via maternal intravenous alcohol binges during the majority of the second trimester. Second, Kelly et al. used a more severe alcohol exposure with neonatal blood alcohol concentrations of 480 ± 22.3 mg/dL (mean $\pm$ SD), compared to our maternal blood alcohol concentrations of 216 ± 20 mg/dL. Third, Kelly et al. quantified microvessel density as capillaries per square millimeter while we estimated microvessel density (microvessels per micron cubed) using unbiased stereology. Counting of microvessel nodes allowed us to take into consideration the branching and connectivity of the microvascular network. Since the microvascular network is a three dimensional structure, the counting of microvessel nodes or branch points, which reflects its interconnectedness, is integral to describing the property and function of this network. However, our estimate of microvessel density may not reflect microvessel growth. Limitations of this study are that we did not measure microvessel diameter, which could be a better measure of growth, and could certainly yield different results. In addition, in areas such as the hippocampus or cerebellum or gray versus white matter, there may be regional differences in microvessel density between the alcohol and saline groups.

3.1. Methodological Considerations

We used the endothelial cell marker, antibody to eNOS (phospho-specific) to label the vessels. In another study in rat brain, immunohistochemical labeling of vessels with antibody to eNOS was identical to labeling with rat pan-endothelial cell marker, RECA-1 (Topel et al., 1998). Other antibodies we tested included anti- von Willebrand factor (vWF), anti-CD31, anti-CD34, and anti-eNOS/NOS type III. We chose the phospho-specific eNOS marker because it more reliably marked vessels compared to the endothelial markers vWF, CD31, and CD34, and did not stain neurons as was found using eNOS/NOS type III. There are developmental differences in the expression of eNOS which could have affected our results. Previous studies in fetal sheep

have shown that eNOS expression in brain parallels the maturation of the cerebrovasculature throughout gestation and that neuronal NOS expression is time, region, and neuronal type dependent (Northington et al., 1996). However, levels of NOS activity equal or exceed adult values by 135 days of gestation in sheep (Northington et al., 1997) and thus we do not believe that developmental differences in eNOS expression affected our results.

We used unbiased stereology to estimate brain microvessel density. It is an efficient method due to the limited sampling needed. We had a low coefficient of error, 0.00–0.15 Cruz-Orive. In contrast to cross-sectional counting, use of unbiased stereology with an optical fractionator design to count nodes and the numbers of microvessel segments joining in those nodes allows the number of microvessels to be estimated, without over counting. Thus, we found it an excellent technique to accomplish this study.

In conclusion, we have shown that 2nd trimester prenatal alcohol exposure in the ovine model does not alter the density of fetal cerebral microvessels. Other mechanisms for the cerebrovascular effects of prenatal alcohol exposure remain to be explored. These include other measures of vessel growth and function as well as possible alcohol-induced alterations in the expression of vasoactive mediators such as adenosine and vasoactive intestinal peptide.

4. Experimental Procedures

4.1. Animals

Twenty near-term (125 ± 1 days gestation (mean $\pm$ SD); term=150 days) fetal sheep brains were examined for this study. The fetuses were the progeny of Polypay ewes and Suffolk rams. The ewes were bred, housed, and received IV alcohol or saline infusions during the second trimester under the auspices of Troy L. Ott, Ph.D., at the animal research facilities of the University of Idaho, Department of Animal and Veterinary Science, Center for Reproductive Biology. All animal procedures were approved by the relevant institution's animal care and use committees (University of Washington; University of Idaho).

4.2. Maternal Infusion Protocol

Pregnant ewes began the infusion protocol at the University of Idaho on day 60 ± 2 of gestation. After pregnancy was confirmed by palpation and ultrasound, a jugular venous catheter was placed percutaneously after administration of Xylazine (0.1 mg/kg i.m.) and local Lidocaine anesthesia. Ewes were allowed to recover for 24 hrs prior to beginning the infusion protocol via this catheter. A long-acting antibiotic, Oxytetracycline (LA-200, 4.1 mg/kg), was administered just prior to catheter placement, at catheter removal, and in the event of elevated temperature. Fifteen ewes were divided into two groups: alcohol-exposed ($n=8$) and saline-control ($n=7$). Ewes in the alcohol group received 1.5 g/kg of pure ethanol diluted 1:3 in normal saline (infusion volume = 5.7 mL/kg) infused daily (Monday-Friday) over a 1.5-hour interval. Ewes in the saline-control group received an equivalent volume of saline (5.7 mL/kg) using the same protocol. Infusions were performed five days per week for a period of four weeks beginning on day 60 ± 2 and ending on day 84 ± 2 of gestation for a total of 19 days of infusion during the 25-day infusion period. Ewes were weighed weekly and infusion volume was adjusted accordingly. The ewes were provided daily with ad lib hay and water and group ration of grain feed. They continued to have access to food during infusions. Measurements of maternal blood alcohol concentrations immediately before and after the infusion in both study groups were obtained on Tuesday and Friday of each infusion week. Blood was drawn into a heparinized syringe by jugular venous puncture from the side of the neck opposite the infusion catheter. Maintenance of pregnancy was confirmed by ultrasonography at the end of the infusion period, and again before transporting the pregnant ewes to the University of Washington two weeks prior to fetal brain removal and study.

4.3. Maternal Measurements

Maternal blood samples obtained pre- and post-infusion were immediately deproteinized, centrifuged, and the supernatants decanted and stored at -20°C for further analysis. Blood alcohol concentrations were measured enzymatically by alcohol dehydrogenase using standard assay kits (Sigma-Aldrich, St. Louis, MO and Diagnostic Chemicals Limited, Oxford, CN).

4.4. Fetal Measurements

Number of fetuses, gender, brain weight, and total body weight were recorded at the time of brain removal.

4.5. Fetal Brain Removal and Preparation

On day 125 ± 1 of gestation the pregnant ewes were killed with an overdose of sodium pentobarbital (90mg/kg) and the fetuses were given sodium pentobarbital (approximately 15mg/kg, diluted 1:3) through the umbilical vein, and then removed from the uterus via hysterotomy. The fetal carotid arteries were cannulated, the jugular veins cut, and the fetal brains perfused with 250 ml heparinized saline (10 units/mL) and then with 250 ml cold 4% paraformaldehyde (PFA) per forebrain hemisphere. The brains were removed, weighed, and immersed in PFA for 1–2 days. The brains were then divided along the falx cerebri and the cerebellum removed. The left forebrain hemisphere was cut at 5mm intervals in the coronal plane, starting at the caudal end and using a plexiglass cutting guide. These slabs were stored in 70% ethanol at 4°C until processed for immunohistochemistry (IHC). The slabs were dehydrated through increasing concentrations of ethanol (70%, 95%, and 100%), processed through xylene and infiltrated with paraffin. The orientation of each tissue slab was maintained so that the caudal aspect was at the surface of each paraffin block. The paraffin embedded tissue slabs were then cut into 5.µm thick sections on a rotary microtome (Leica), the sections were mounted on Superfrost plus slides, air dried, baked in an oven at 60°C for 1 hour, and then stored at room temperature. From each block we took a section at the point that the embedded tissue slab was complete. Since the sections from each block were taken in the same manner (thus approximately the same place in the slab), this established a section interval equal to the thickness of the slab (5mm), or every 1000th 5µm section. In this sampling method there should be no bias in selecting the sections for staining and analysis. Additional sections were used for IHC controls.

4.6. Immunohistochemistry

To identify vessels in the fetal sheep brain, we labeled vascular endothelial cells with primary antibody to eNOS using an avidin-biotin peroxidase IHC method. The ten sections from each brain were deparaffinized in Citrisolv and rehydrated in a series of graded ethanol concentrations (100%, 95%, 80%, and 70%) and H_2O . Antigen retrieval was done by microwave heating the sections to boiling in 10mM citric acid buffer (pH 6.0) at high power (100%) for 4 minutes then at low power (20%) for 9 minutes followed by cooling for 30 minutes. The sections were washed in 0.05M PBS, then 0.1M PBS. Endogenous peroxidase activity was blocked by immersing the sections in 3% hydrogen peroxide in 0.1M PB. Sections were washed in 0.05M PBS and then incubated with working solution (0.05M PBS + 0.3% TX-100 + 1% BSA + 3% horse serum) to reduce background staining. The sections were then incubated overnight (~20 hours) with the primary antibody, phospho-specific mouse anti-eNOS (BD Transduction Laboratories, Franklin Lakes, NJ, USA), diluted 1:500 in working solution at room temperature followed by washing in 0.05M PBS. The sections were then incubated for 1 hour with the biotinylated horse anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, USA), diluted 1:250, followed by washing in 0.05M PBS. The sections were then incubated for 30 minutes with avidin-biotin complex diluted 1:100 (Vector Laboratories, Burlingame, CA, USA), and then washed in 0.05M PBS followed by a wash in

0.1M Tris buffer. The sections were developed in 0.25 mg/ml DAB in 0.1M Tris buffer, pH 7.6 with μ l 3% H₂O₂ /ml for 7.5 minutes. Sections were washed in 0.1M Tris Buffer, then 0.05 PBS, and finally with deionized H₂O. The sections were dehydrated through ascending alcohols (70%, 80%, 95%, 100%, cleared in Citrisolv, and mounted under glass coverslips using Protocol mounting medium (Fisher Diagnostics, Middletown, VA, USA).

4.7. Stereology

Within the microvascular network, nodes are the sites where microvessels meet. The valence of a node is the number of microvessels that join in that node. Lokkegaard (2004) calculated the number of microvessels based on the correlation between the number of microvessels and the number of nodes in which they meet and the valences of those nodes. Therefore we counted microvessel nodes, classifying each node based on its valence.

We quantified microvessel density in the immunohistochemically labeled sections using light microscopy and hardware/software designed for unbiased stereology: A Leitz Diaplan microscope was used with a 100 x, 1.4 numerical aperture oil immersion objective, computer-driven stage (Ludl Electronic Products) and a microcator (Heidenhain, Germany). Microvessel nodes were counted with the aid of StereoInvestigator® version 6 (Microbrightfield, Williston, VT, USA), a semi-automated computer program that manages the tissue sections, controls the position of the microscope stage, records counted nodes of different valences, and then estimates the total number of nodes in the structure of interest. We counted microvessel nodes in a systematically random sampled known fraction of the entire left forebrain hemisphere using an optical fractionator (optical disectors in a fractionator sampling design) and applying unbiased counting rules. Prior to counting, the outer edge of each forebrain hemisphere tissue section was outlined within the StereoInvestigator® program at 2.5x magnification to create the region of interest.

In order for estimates of number of microvessel nodes to be accurate, any given node must have the possibility of being counted only once. Therefore, strict counting criteria were established prior to performing stereologic estimates of numbers of microvessel nodes. Nodes densely labeled with brown reaction product were counted if their microvessels were less than 10 μ m in diameter, if the node did not touch the inferior or left vertical borders of the counting frame, and if the node came into focus as the counting frame was moved down through the height of the section thickness. Details of stereological counting techniques have been described (West and Gundersen, 1990; West, 2002).

An optical fractionator survey was performed as part of a pilot study to determine the best parameters. Counting frame size and distribution were based on density and distribution of nodes obtained in a preliminary population estimate. The coefficient of error (CE), or uncertainty of the estimate, was evaluated to determine that the appropriate design parameters were chosen. Design parameters remained constant for any one forebrain hemisphere. Using StereoInvestigator®, 60 $\times$ 90 μ m counting frames were placed at sites on a 2000 $\times$ 2500 μ m counting grid, and observations were made at each site. Approximately 700 individual sites were sampled for each forebrain hemisphere, using a random starting point. Approximately 35 immunopositive nodes were counted in each left forebrain hemisphere. From these counts, StereoInvestigator generated an estimate of total number of nodes of each valence in each left forebrain hemisphere. The CE (Cruz-Orive) for each brain counted was 6.5% (mean $\pm$ SD). All eNOS-immunostained brains were counted in a blinded fashion by a single observer (KS).

4.8. Data Analysis

Microvessel density in each left cerebral forebrain hemisphere was estimated by dividing the calculated number of microvessels in the volume of the disectors by the sum of the volume of the disectors, according to the method of Lokkegaard (2004).

$$\text{Estimated Microvessel Density} = \frac{\sum [(n-2)/2 \times P_{n+1}]}{\sum v(\text{dis})}$$

n = valence, or number of branches

P_n = number of nodes of valence n

v(dis) = volume of the disectors = height of disector × area of counting frame × number of sampling sites in one tissue section

The mean microvessel density for alcohol-exposed and saline-control groups were calculated for the left forebrain hemispheres of each group, and Mann Whitney rank sum test was used to determine statistical significance. The alpha level was set at 0.05 for all tests. There is one outlier data point in the alcohol-exposed group. We have no basis for excluding this animal on technical or statistical grounds or measured maternal or fetal parameters. Even if the outlier were to be excluded, we would still not be able to show a statistically significant difference in microvessel density between the experimental and control groups (Fig. 2).

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References

- American Academy of Pediatrics Committee on Substance Abuse and Committee on Children with Disabilities. Fetal alcohol syndrome and alcohol-related neurodevelopmental disorders. *Pediatrics* 2000;106:358–361. [PubMed: 10920168]
- Detering N, Collins RM Jr, Hawkins RL, Ozand PT, Karahasan A. Comparative effects of ethanol and malnutrition on the development of catecholamine neurons: a long-lasting effect in the hypothalamus. *J. Neurochem* 1981;36:2094–2096. [PubMed: 7241151]
- Dobbing J, Sands J. Comparative aspects of the brain growth spurt. *Early Hum. Dev* 1979;3:79–83. [PubMed: 118862]
- Gleason CA, Iida H, Hotchkiss KJ, Northington FJ, Traystman RJ. Newborn cerebrovascular responses after first trimester moderate maternal ethanol exposure in sheep. *Pediatr. Res* 1997;42:39–45. [PubMed: 9212035]
- Goodlett CR, Leo JT, O'Callaghan JP, Mahoney JC, West JR. Transient cortical astrogliosis induced by alcohol exposure during the neonatal brain growth spurt in rats. *Brain Res. Dev. Brain Res* 1993;72:85–97.
- Gordon BH, Streeter ML, Rosso P, Winick M. Prenatal alcohol exposure: abnormalities in placental growth and fetal amino acid uptake in the rat. *Biol. Neonate* 1985;47:113–119. [PubMed: 3978155]
- Hammer RP Jr, Scheibel AB. Morphologic evidence for a delay of neuronal maturation in fetal alcohol exposure. *Exp. Neurol* 1981;74:587–596. [PubMed: 7297637]
- Jones KL, Smith DW, Ulleland CN, Streissguth P. Pattern of malformation in offspring of chronic alcoholic mothers. *Lancet* 1973;1(7815):1267–1271. [PubMed: 4126070]
- Kelly SJ, Mahoney JC, West JR. Changes in brain microvasculature resulting from early postnatal alcohol exposure. *Alcohol* 1990;7:43–47. [PubMed: 2310504]

- Lokkegaard, A. The number of microvessels estimated by an unbiased stereological method applied in a brain region. In: Evans, SM.; Janson, AM.; Nyengaard, JR., editors. *Quantitative Methods in Neuroscience: A Neuroanatomical Approach*. New York: Oxford University Press Inc.; 2004. p. 167-182.
- Mayock DE, Ness D, Mondares RL, Gleason CA. Binge alcohol exposure in the second trimester attenuates fetal cerebral blood flow response to hypoxia. *J. Appl. Physiol* 2007;102:972-977. [PubMed: 17341736]
- Miller MW. Effects of alcohol on the generation and migration of cerebral cortical neurons. *Science* 1986;233:1308-1311. [PubMed: 3749878]
- Miller MW, Potempa G. Numbers of neurons and glia in mature rat somatosensory cortex: effects of prenatal exposure to ethanol. *J. Comp. Neurol* 1990;293:92-102. [PubMed: 2312794]
- Morgane PJ, Austin-LaFrance R, Bronzino J, Tonkiss J, Diaz-Cintra S, Cintra L, Kemper T, Galler JR. Prenatal malnutrition and development of the brain. *Neurosci. Biobehav. Rev* 1993;17:91-128. [PubMed: 8455820]
- Northington FJ, Koehler RC, Traystman RJ, Martin LJ. Nitric oxide synthase 1 and nitric oxide synthase 3 protein expression is regionally and temporally regulated in fetal brain. *Brain Res. Dev. Brain Res* 1996;95:1-14.
- Northington FJ, Tobin JR, Harris AP, Traystman RJ, Koehler RC. Developmental and regional differences in nitric oxide synthase activity and blood flow in the sheep brain. *J. Cereb. Blood Flow Metab* 1997;17:109-115. [PubMed: 8978393]
- Nowoslawski L, Klocke BJ, Roth KA. Molecular regulation of acute ethanol-induced neuron apoptosis. *J. Neuropathol. Exp. Neurol* 2005;64:490-497. [PubMed: 15977640]
- Pierce DR, West JR. Blood alcohol concentration: a critical factor for producing fetal alcohol effects. *Alcohol* 1986;3:269-272. [PubMed: 3638973]
- Rosett HL, Weiner L, Lee A, Zuckerman B, Dooling E, Oppenheimer E. Patterns of alcohol consumption and fetal development. *Obstet. Gynecol* 1983;61:539-546. [PubMed: 6835605]
- Sowell ER, Mattson SN, Thompson PM, Jernigan TL, Riley EP, Toga AW. Mapping callosal morphology and cognitive correlates: effects of heavy prenatal alcohol exposure. *Neurology* 2001;57:235-244. [PubMed: 11468307]
- Streissguth AP, Aase JM, Clarren SK, Randels SP, LaDue RA, Smith DF. Fetal alcohol syndrome in adolescents and adults. *J. Am. Med. Assoc* 1991;265:1961-1967.
- Topel I, Stanarius A, Wolf G. Distribution of the endothelial constitutive nitric oxide synthase in the developing rat brain: an immunohistochemical study. *Brain Research* 1998;788:43-48. [PubMed: 9554947]
- West JR, Chen WJ, Pantazis NJ. Fetal alcohol syndrome: the vulnerability of the developing brain and possible mechanisms of damage. *Metab. Brain Dis* 1994;9:291-322. [PubMed: 7898398]
- West MJ. Design-based stereological methods for counting neurons. *Prog. Brain Res* 2002;135:43-51. [PubMed: 12143362]
- West MJ, Gundersen HJ. Unbiased stereological estimation of the number of neurons in the human hippocampus. *J. Comp. Neurol* 1990;296:1-22. [PubMed: 2358525]

Figure 1.
Fetal sheep forebrain cortex. Vessels are stained brown by the eNOS immunoreaction product.
Arrows indicate microvessel nodes, with valence in parentheses.
1A. Low magnification: Scale bar is 200 μm.
1B. High magnification: Scale bar is 50μm.

Figure 2.

Fetal sheep forebrain microvessel density estimates (microvessels per mm³). The mean fetal forebrain microvessel density estimate was 12.7 ± 8.7 (mean $\pm$ SD) microvessels per mm³ in the alcohol-exposed fetuses and 9.1 ± 2.8 (mean $\pm$ SD) microvessels per mm³ in the saline-controls ($p = 0.32$).

Table 1

Baseline data for 20 fetal sheep.

	Sex	Brain wt (g)	Body wt (g)	Brain wt. as % of body wt.
Alcohol-Exposed	2 M, 7 F	44.4 ± 3.1	3389 ± 472	1.33 ± 0.18*
Saline-Control	6 M, 5 F	45.4 ± 2.0	3018 ± 426	1.53 ± 0.18

Values are expressed as mean ± SD.

*
 $p=0.02$ compared to control animals.