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THIRD TRIMESTER-EQUIVALENT ETHANOL EXPOSURE CAUSES MICRO-HEMORRHAGES IN THE RAT BRAIN

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Abstract—Exposure to ethanol during fetal development produces long-lasting neurobehavioral deficits caused by functional alterations in neuronal circuits across multiple brain regions. Therapeutic interventions currently used to treat these deficits are only partially efficacious, which is a consequence of limited understanding of the mechanism of action of ethanol. Here, we describe a novel effect of ethanol in the developing brain. Specifically, we show that exposure of rats to ethanol in vapor chambers during the equivalent to the third trimester of human pregnancy causes brain micro-hemorrhages. This effect was observed both at low and high doses of ethanol vapor exposure, and was not specific to this exposure paradigm as it was also observed when ethanol was administered via intra-esophageal gavage. The vast majority of the micro-hemorrhages were located in the cerebral cortex but were also observed in the hypothalamus, midbrain, olfactory tubercle, and striatum. The auditory, cingulate, insular, motor, orbital, retrosplenial, somatosensory, and visual cortices were primarily affected. Immunohistochemical experiments showed that the micro-hemorrhages caused neuronal loss, as well as reactive astrogliosis and microglial activation. Analysis with the Catwalk test revealed subtle deficits in motor function during adolescence/young adulthood. In conclusion, our study provides additional evidence linking developmental ethanol exposure with alterations in the fetal cerebral vasculature. Given that this effect was observed at moderate levels of ethanol exposure, our findings lend additional support to the recommendation that women abstain from consuming alcoholic beverages during pregnancy.

Key words: hemorrhage, fetal, ethanol, development, neonatal, vascular.

INTRODUCTION

Among the environmental factors that cause intellectual disability, a leading one across the globe is exposure of the fetus to ethanol. The effects of ethanol range from isolated neurobehavioral disorders to a combination of morphological and intellectual abnormalities that characterize Fetal Alcohol Syndrome. Collectively, these are known as Fetal Alcohol Spectrum Disorders (FASDs). FASDs are a prevalent condition that affects children both in developing and developed nations (Hutson et al., 2010; May et al., 2013, 2014; Chambers et al., 2014). Studies have shown that ethanol has a myriad of effects on the developing brain, including alterations in neuron and glial cell differentiation, migration, and survival (Luo, 2009; de la Monte and Kril, 2014), as well as deficits in the formation and refinement of synapses, and long-term impairments in several neurotransmitter systems (Medina, 2011; Valenzuela et al., 2011). Multiple mechanisms are thought to be responsible for these effects; for example, excitotoxicity, endocrine disturbances, oxidative stress, epigenetic alterations, over-activation of the neuroimmune system, as well as cell adhesion molecule and neurotrophic factor dysfunction (Goodlett et al., 2005; Bekdash et al., 2014; Drew and Kane, 2014).

In addition to the mechanisms mentioned above, it has been shown that cardiovascular alterations can mediate the effects of ethanol on the developing brain (reviewed in (Ramadoss and Magness, 2012). Binge exposure of ewes to relatively high levels of ethanol during the equivalent to the second trimester of human pregnancy blunted the responsiveness of the fetal cerebral vasculature to hypoxia later in pregnancy (Mayock et al., 2007). Similarly, exposure of ewes to high (but not low) doses of ethanol in a binge-like manner during the last trimester of pregnancy induced fetal tachycardia, hypotension, acidemia, and hypercapnia, as well as increased blood flow in the cerebellum, an effect that was associated with loss of Purkinje neurons (Cudd et al., 2001; Parnell et al., 2007; Kenna et al., 2011). Kenna et al. (2011) reported the presence of small subarachnoid hemorrhages in the forebrain and cerebellum in approximately 40% of fetal sheep exposed to lower levels of ethanol during late pregnancy. In a subsequent study from the same group of investigators, it was reported that this ethanol exposure paradigm increased stiffness of arteries in several fetal organs, including the brain (Parkington et al., 2014). Jegou et al. (2012) demonstrated that second trimester-equivalent exposure of mice

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Abbreviations: ANOVA, analysis of variance; DAPI, 4′,6-diamidino-2-phenylindole; FASDs, Fetal Alcohol Spectrum Disorders; GFAP, glial fibrillary acidic protein; IBA-1, ionized calcium-binding adapter molecule 1; NMDA, N-methyl-D-aspartate; NeuN, neuronal nuclei; P, postnatal day; PFA, paraformaldehyde; PBS, phosphate-buffered saline.

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to high levels of ethanol decreased the density and altered the orientation of blood vessels in the cerebral cortex via a mechanism that involves reduced expression of vascular endothelial growth factor and inhibition of N-methyl-D-aspartate (NMDA) receptor-mediated increases in intracellular Ca^{2+} levels in endothelial cells. These investigators also demonstrated alterations in the radial orientation of small blood vessels in the human fetal cortex at gestational weeks 30–38. Wang et al. (2015) recently showed that ethanol can inhibit angiogenesis in chick embryos, an effect that could be mediated by a reduction in expression of genes involved in this process, including vascular endothelial growth factor. Collectively, these studies indicate that alterations in the developing cerebral vasculature play an important role in the pathophysiology of FASDs.

During the first week of life in rats (equivalent to the third trimester of human gestation; Clancy et al., 2007), studies from a number of laboratories have demonstrated that developing cerebral micro-vessels are particularly susceptible to rupture in response to insults, such as exposure to ionizing radiation and increases in vascular tone (Landolt and Arn, 1979; Pavlik and Mares, 1992; Pahlavan et al., 2012). Based on these studies and the fact that ethanol can induce vasoconstriction (Altura et al., 1983; Liu et al., 2004), we hypothesized that ethanol exposure during this period of development could induce rupture of brain micro-vessels. We found that exposure to high levels of ethanol administered in a binge-like fashion did induce micro-hemorrhages in several brain regions. This effect was also observed in rats exposed to more moderate doses of ethanol and was subsequently associated with cell death and reactive gliosis.

EXPERIMENTAL PROCEDURES

Ethanol exposure paradigms

The Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center approved all procedures. Pregnant Sprague–Dawley rats were obtained from Harlan (Indianapolis, IN, USA). Rats arrived between gestational days 14 and 16 to allow them to acclimate to our Animal Research Facility for 5–7 days before giving birth. Both male and female offspring were used for all experiments. To model exposure during a period of rat brain development that coincides with the third trimester of human gestation (Clancy et al., 2007), neonates and dams were exposed to ethanol in vapor inhalation chambers on postnatal days (P) 3, P4, and/or P5 with controls concomitantly exposed to only air in identical chambers. High dose ethanol exposure (ethanol vapor concentration ~8 g/dl, 3 h/day; pup blood ethanol levels ~0.4 g/dl at P4; maternal blood ethanol levels ~0.02 g/dl) was performed in custom-built chambers, as previously described (Baculis et al., 2015). This exposure paradigm is associated with a low pup mortality rate (0.65%), as previously reported (Baculis et al., 2015). Given that maternal blood ethanol concentrations were low, additional exposure of pups to ethanol via breast milk is expected to be negligible; blood ethanol levels in suckling pups have been shown to be approximately 10% of those of lactating dams consuming ethanol (Barbier et al., 2009). We measured O_2 levels in the ethanol chambers using a fiber-optic O_2 meter (World Precision Instruments, Sarasota, FL, USA) and, as expected, we found O_2 levels to be 92% of those in both ambient air and inside the air control chambers. When indicated, pups and dams were exposed to a lower ethanol vapor concentration of ~3 g/dl for 3 h/day, resulting in peak pup blood ethanol levels of 0.086 ± 0.01 g/dl (n = 5; measured with alcohol dehydrogenase assay at P4, as previously described; Galindo and Valenzuela, 2006). As a control, 24-day-old juvenile rats were exposed to ~8 g/dl ethanol vapor for 4 h/day for 3 days (peak blood ethanol level = 0.29 ± 0.01, n = 3). For some experiments, a 30% w/v ethanol solution mixed with intralipid 20% (Baxter, Deerfield, IL, USA) or intralipid 20% alone were administered to rat pups by intra-esophageal gavage on P3–P5 (daily ethanol dose = 5.25 g/kg; blood ethanol levels = 0.36 g/dl ± 0.022 at P3, 2 h after gavage; n = 6).

Quantification of micro-hemorrhages

Rats were anesthetized with isoflurane (Piramal Healthcare, Andhra Pradesh, India) and euthanized by rapid decapitation. Brains were carefully removed from the skull and fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) at 4 °C for 48 h and then stored in PBS at 4 °C. Whole-brain surface imaging was accomplished using a dissecting microscope (SZH Zoom Stereo Microscope System, Olympus, Center Valley, PA, USA) equipped with a 7.5× numerical aperture objective and a Motic 2300 3.0MP digital camera connected to a PC computer running Motic Images Plus 2.0 software (Motic, Hong Kong, China). Surface bleeds were quantified using a custom-built, blinded counting application written in Personal Home Page Hypertext Preprocessor, Javascript, and HyperText Markup Language. To determine the regional distribution of the micro-bleeds, 250-μm coronal brain sections were prepared using a vibrating slicer (Vibratome Series 1000, Technical Products International, Inc., St. Louis, MO). Sections were subsequently examined with a convergent objective ZSB stereo microscope (Unitron, Commack, NY, USA).

Immunohistochemistry

Rats were deeply anesthetized with ketamine (250 mg/kg I.P.) and transcardially perfused at a speed of 0.03 ml/min (0.457 mm × 1.3 cm standard hypodermic needles; Covidien, Mansfield, MA, USA; 60 Hz variable flow peristaltic pump; Thermo Fisher Scientific, Waltham, MA, USA) for 3 min with PBS containing procaine hydrochloride (1 g/l; Sigma–Aldrich, St. Louis, MO, USA) and heparin (1 USP unit/l, Becton, Dickinson and Company, Franklin Lakes, New Jersey, USA) at 37 °C, followed by perfusion for 1.5 min with 4% PFA at 22 °C, and finally, perfusion for 3 min with ice-cold 4% PFA. Following decapitation, brains were removed from the skull and fixed in 4% PFA at 4 °C for 48 h and then incubated in 30% sucrose in PBS until the tissue sank