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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.

Keywords

hemorrhage; fetal; ethanol; development; neonatal; vascular

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Authors Contributions were as follows: JHW performed the experiments, analyzed data, and helped write the manuscript; JJM performed experiments, wrote custom computer software for micro-hemorrhage quantification, and analyzed data; ALL performed the gavage experiments and data analysis; BCB performed the Catwalk experiments and analyzed the data; CFV assisted with experiments, analyzed data, supervised the project, designed the experiments, and wrote the manuscript.

1. 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, Chambers et al., 2014, May 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 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.

2. EXPERIMENTAL PROCEDURES

2.1. 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). 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 hr/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 O₂ levels in the ethanol chambers using a fiber-optic O₂ meter (World Precision Instruments, Sarasota, FL) and, as expected, we found O₂ 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 hr/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 hr/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) or intralipid 20% alone were administered to rat pups by intra-esophageal gavage on P3–5 (daily ethanol dose = 5.25 g/kg; blood ethanol levels = 0.36 g/dl ± 0.022 at P3, 2 hr after gavage; n = 6).

2.2. 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 hr 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) equipped with a 7.5x 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 μ m 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).

2.3. 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 $\times$ 1.3 cm standard hypodermic needles; Covidien, Mansfield, MA; 60Hz variable flow peristaltic pump; Thermo Fisher Scientific, Waltham, MA) for 3 min with PBS containing procaine hydrochloride (1 g/l; Sigma-Aldrich, St. Louis, MO) and heparin (1 USP unit/l, Becton, Dickinson and Company, Franklin Lakes, New Jersey) at 37 $^{\circ}$ C, followed by perfusion for 1.5 min with 4% PFA at 22 $^{\circ}$ C, and finally, perfusion for 3 minutes with ice-cold 4% PFA. Following decapitation, brains were removed from the skull and fixed in 4% PFA at 4 $^{\circ}$ C for 48 hr and then incubated in 30% sucrose in PBS until the tissue sank to the bottom of the tube. Blocks containing the somatosensory and motor cortices were immersed in Optimal Cutting Temperature compound (Fisher Healthcare, Houston, TX) and frozen in 2-methylbutane cooled in a dry ice/95% ethanol bath. Frozen tissue was stored at -80° C in sealed plastic bags. Tissue sections (thickness = 14 μ m) were prepared with a cryostat (model HM 505E, Microm International, Walldorf, Germany) and placed on Superfrost Plus micro slides (VWR International, Radnor, PA). Frozen tissue sections were stored at -20° C in sealed plastic bags.

On the day of immunohistochemical experiments, slides were allowed to dry at room temperature for 30 min and a hydrophobic barrier was drawn around the tissue using a PAP pen (Diagnostic BioSystems, Pleasanton, CA). Tissue sections were incubated for 1.5 hr at 22 $^{\circ}$ C with PBS containing 1% bovine blood albumin (Sigma-Aldrich, St. Louis, MO), 0.2% Triton X-100 (Sigma-Aldrich, St. Louis, MO), and 5% donkey and goat serum (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Subsequently, sections were incubated with mouse antiglial fibrillary acidic protein (GFAP) monoclonal antibody (1:200, catalog number: G3893, Sigma-Aldrich, St. Louis, MO), mouse anti-neuronal nuclei (NeuN) monoclonal antibody (1:200, catalog number: MAB377, EMD Millipore, Billerica, MA), rabbit anti-ionized calciumbinding adapter molecule 1 (IBA-1) polyclonal antibody (1:200, catalog number: Z0334, Wako, Richmond, VA), and/or 4',6-diamidino-2-phenylindole (DAPI) (1:4000, Life Technologies, Carlsbad, CA). Immunolabeling was detected by incubating for 1.5 hr at 22 $^{\circ}$ C with donkey anti-rabbit Alexa Fluor 555 (1:1000, catalog number: A31572, Invitrogen, Waltham, MA), donkey anti-mouse Alexa Fluor 555 (1:1000, catalog number: A31570, Invitrogen), or goat anti-mouse Alexa Fluor 488 (1:1000, catalog number: A11029, Invitrogen) secondary antibodies. Sections were mounted with Vectashield antifade medium (Vector Laboratories, Burlingame, CA) then imaged with a Leica TCS-SP8 Confocal Microscope & Fluorescence Lifetime Imaging System (Leica Microsystems,

Buffalo Grove, IL) equipped with a tunable, pulsed white light laser (470–670 nm), 405 nm diode laser, and a 20x 0.75 numerical aperture oil immersion objective. Blue, red, and green fluorescence were detected using prism-based emission fluorescence separation to sequentially detect predetermined emission spectrums. Background-subtracted Z-stack compressed images were analyzed using ImageJ/Fiji software (Schindelin et al., 2012). A standardized region of interest was then set at 50 μm height $\times$ 400 μm width centered over the micro-hemorrhage site at an angle perpendicular to its longest axis. Each data point was normalized to the average of the first and last 50 data points in the region of interest. The number of micro-hemorrhages analyzed per brain were 9 ± 2.2 for the GFAP staining and 6.5 ± 0.86 for the NeuN stained sections (15–19 sections/brain; $n = 4$ brains each from a different litter). The number of micro-hemorrhages analyzed per brain were 7.6 ± 1.7 for the microglia staining; the number of fields analyzed for the micro-hemorrhage free areas in the brains of ethanol exposed pups was 4.3 ± 0.67 for the brains of air exposed animals was 3.6 ± 0.66 (10–13 sections/brain; $n = 3$ brains each from a different litter).

2.4. Analysis of Motor Function

Locomotor activity and gait were assessed at P48 and P71 using the Catwalk XT system (Noldus, Leesburg, VA). We chose this test because it has been previously used to characterize the impact of ischemic and hemorrhagic stroke on motor function in rats (Encarnacion et al., 2011). Rats were placed in the entrance of a 1.3 m-long glass walkway, which was connected to a dark goal box and enclosed by an adjustable tunnel. Animals were allowed to walk freely across the runway to reach the goal box containing food and bedding from their home cages. Green fluorescence light on the long edge of the glass plate illuminated paw prints when a paw made contact with the glass. The ceiling of the tunnel was illuminated by red light to allow detection of the animal's silhouette. Paw prints and silhouettes were captured by a digital high-speed video-camera. Three to four compliant walkway trials, defined as crossing the entire walkway without stopping or turning around during a timeframe of 0.5 to 10 s with maximum body speed variation less than 60%, were recorded for each rat. The average run duration was 5.3 ± 0.43 s and 4.66 ± 0.28 s for the control and ethanol groups, respectively ($t(28)=1.22$; $P = 0.23$ by unpaired t-test; $n = 15$ for both groups). The total number of trials required to achieve 3–4 compliant trials was 5.8 ± 0.95 and 6 ± 0.96 for the control and ethanol groups, respectively ($U = 109.5$; $P = 0.9$ by Mann-Whitney test; $n = 15$ for both groups). After each trial, the walkway was thoroughly cleaned with 70% ethanol and deionized water. Catwalk XT 8.1 software (Noldus) was used to analyze the data.

2.5. Statistical Analysis

Prism 6.0 (GraphPad Software, San Diego, CA) was used for all statistical analyses. The level of significance was $p < 0.05$. The unit of determination (n) was the average of results obtained with all samples from an animal. Whether the data followed a normal distribution was determined using the Kolmogorov-Smirnov test. Data that passed the normality test were analyzed by a two-tailed t-test. Data that did not pass the Kolmogorov-Smirnov test were analyzed by the Mann-Whitney test. When indicated, data were analyzed by two-way analysis of variance (ANOVA) followed by Bonferroni's posthoc test.

3. RESULTS

3.1. Third trimester-equivalent ethanol exposure causes micro-hemorrhages in the surface of the developing brain

Fig 1A illustrates the third trimester-equivalent ethanol vapor chamber exposure paradigm and the times at which brains were collected for analyses. We exposed pups and dams to high levels of ethanol (pup blood ethanol levels near 0.4 g/dl) for 3 hr/day between P3 and P5. On samples collected at the end of chamber exposure on the first day of the paradigm (P3; Exposure 1; Fig 1A), we detected a few spontaneous micro-hemorrhages on the surface of brains from control rats and a similar number of micro-hemorrhages in samples from the ethanol group (Fig 1B; $t(10) = 0.68$; $p = 0.51$). The morning after, we collected samples prior to the start of the second day of exposure (P4; Exposure 2; Fig 1A) and observed a significant (approximately 7-fold) increase in the average number of surface micro-hemorrhages in brains from the ethanol group (Fig 1B; $t(18) = 5.43$; $p < 0.0001$). A similar result was obtained with samples collected the morning after the last day of the paradigm (P6; Fig 1A), where ethanol exposure induced approximately a 5-fold increase in the average number of surface micro-hemorrhages (Fig 1B; Mann-Whitney $U = 59$; $p < 0.0001$); the size of the micro-hemorrhages was $9.2 \pm 1.1 \mu\text{m}^2$ for controls ($n = 28$ pups from 6 litters) and $28.2 \pm 1.8 \mu\text{m}^2$ for ethanol ($n = 26$ pups from 6 litters) groups ($t(52) = 8.72$; $P < 0.0001$). At P6, the effect of ethanol on the average number of micro-hemorrhages/brain was similar in male (control = 6.2 ± 2.5 , ethanol = 21 ± 3.5 , $n = 9$ pups from 4 litters) and female animals (control = 3.3 ± 0.5 , ethanol = 17.5 ± 3.0 , $n = 16$ pups from 5 litters) (two-way ANOVA; sex: $F(1, 46) = 1.47$; $P = 0.23$; treatment: $F(1, 46) = 30.78$; $P < 0.0001$; interaction: $F(1, 46) = 0.01$; $P = 0.92$; posthoc test: $P < 0.05$ control vs. ethanol both in males and females). Examples of brains from control and ethanol exposed animals illustrating the morphological characteristics of the micro-hemorrhages in P6 rats are shown in Fig 1C. A control experiment revealed that the micro-hemorrhages were still visible on P7 but their numbers decreased on P8. We were unable to detect the micro-hemorrhages between P9 and P16 (data not shown).

To determine if the effects of ethanol were dose dependent, we exposed pups and dams to lower levels of ethanol following the same timeline illustrated in Fig 1A (pup blood ethanol levels near the legal intoxication limit of 0.08 g/dl). On samples collected the morning after the last day of the paradigm (P6; Fig 1A), we observed a significant increase (approximately 3-fold) in the average number of micro-hemorrhages in brains from ethanol-treated pups (Fig 2A-B; Mann-Whitney $U = 70$; $p < 0.0001$). To investigate if the increase in micro-hemorrhages was specific to the ethanol vapor chamber paradigm, we exposed pups to ethanol from P3 to P5 via intra-esophageal gavage (pup blood ethanol levels near 0.4 g/dl). Ethanol exposure using this paradigm also caused a significant increase (approximately 5-fold) in the average number of micro-hemorrhages at P6 (Fig 2C-D; Mann-Whitney $U = 13$; $p < 0.0001$). To assess whether the effect of ethanol was age-dependent, we exposed juvenile rats (P21-25) to ethanol vapor for 4 hr/day for 3 days (blood ethanol levels near 0.3 g/dl) and found no evidence of brain surface micro-hemorrhages the day after the end of the 3-day exposure paradigm in rats from both treatment groups (Fig 2E-F; $U = 622$; $p < 0.75$ by Mann-Whitney test).

3.2. Micro-hemorrhages are predominantly located in selected brain regions

The location of the micro-hemorrhages was evaluated in coronal sections from the brains of air- and ethanol-exposed pups. Pups and dams were exposed to a high dose of ethanol (pup blood ethanol levels near 0.4 g/dl) for 3 hr/day on P3, P4 and P5 and brains were collected at P6. In brains from control rats, spontaneous micro-hemorrhages were detected in several brain regions, with the majority of these being located in the cerebral cortex (Table 1). Ethanol significantly increased the number of micro-hemorrhages, not only in the cerebral cortex, but also in the hypothalamus, midbrain, olfactory tubercle, and striatum (Table 1). Further analyses of the micro-hemorrhages in the cerebral cortex revealed that ethanol exposure significantly increased the number of micro-hemorrhages in the auditory, cingulate, insular, motor, orbital, retrosplenial, somatosensory, and visual cortices (Table 2).

3.3. Micro-hemorrhages cause neuronal loss and activation of astrocytes and microglia

We further characterized the cortical micro-hemorrhages using immunohistochemical techniques. Immunostaining with antibodies against the neuronal nuclear marker NeuN confirmed that the micro-hemorrhages cause a significant decrease in neuronal numbers (Fig 3A). We also detected a significant increase in the intensity of immunostaining for the astrocytic marker, GFAP (Fig 3B). In addition, immunostaining with antibodies against the microglial marker, IBA-1, revealed a significant increase in the number of microglia with amoeboid and transitional morphology in the micro-hemorrhages present in brains from the ethanol group (Fig 4; repeated-measures two-way ANOVA; microglia morphology: $F(2, 12) = 6.586$; $P = 0.011$; treatment: $F(2, 6) = 34.71$; $P = 0.0005$; interaction: $F(4, 12) = 34.76$; $P < 0.0001$; posthoc test: $P < 0.05$ micro-hemorrhage zone in ethanol group vs. controls). Using these histological markers, we were unable to determine if the hemorrhages resulted in persistent alterations in brain tissue, as it was difficult to distinguish sites of damage from regions undergoing normal developmentally-related morphological changes.

3.4. Ethanol exposure minimally affects locomotor activity or gait

To characterize the impact of ethanol on motor function, we assessed performance in the Catwalk test at P48-P71. Neither the average body speed (Fig 5A) nor the step cadence (Fig 5B) were significantly affected by ethanol exposure (Speed: $U = 107$; $P = 0.82$ by Mann-Whitney test; Cadence: $t(28) = 0.73$; $P = 0.46$ by unpaired t-test). Two-way ANOVA revealed that ethanol significantly reduced the maximum contact area of the paw (Fig 5C; two-way ANOVA: interaction $F(3, 112) = 0.27$, $P = 0.84$; paw: $F(3, 112) = 0.64$, $P = 0.58$; ethanol treatment $F(1, 112) = 4.41$, $P = 0.03$). However, posthoc analysis with Bonferroni's test did not reveal any significant differences between treatment groups for each paw. A similar result was observed for step cycle (Fig 5D; two-way ANOVA: interaction $F(3, 112) = 0.15$, $P = 0.92$; paw: $F(3, 112) = 0.19$, $P = 0.89$; ethanol treatment $F(1, 112) = 3.99$, $P = 0.04$; Bonferroni's posthoc test: $P > 0.05$ for all paws). Ethanol exposure did not significantly affect either swing speed (Fig 5E; two-way ANOVA: interaction $F(3, 112) = 0.72$, $P = 0.53$; paw: $F(3, 112) = 0.18$, $P = 0.9$; ethanol treatment $F(1, 112) = 8.35 \times 10^{-6}$, $P = 0.99$) or stride length (Fig 5F; two-way ANOVA: interaction $F(3, 112) = 0.22$, $P = 0.88$; paw: $F(3, 112) = 0.64$, $P = 0.58$; ethanol treatment $F(1, 112) = 0.7$, $P = 0.4$). Additional

Catwalk parameters are shown in Table 3, indicating that ethanol exposure significantly reduced the number of steps.

5. DISCUSSION

We report here that ethanol exposure during a period of rat brain development that includes events that take place in the third trimester of human pregnancy causes micro-hemorrhages in the brain of neonatal rats. The presence of spontaneous micro-hemorrhages has been previously documented in the cerebral cortex of naïve neonatal rats (Landolt and Arn, 1979, Pavlik and Mares, 1992). The spontaneous micro-hemorrhages predominantly occurred between P3 and P6, and were mainly found in the middle layers of the cerebral cortex where penetrating arterioles branch into capillaries (Pavlik and Mares, 1992). In agreement with these reports, we detected the presence of micro-hemorrhages in the surface of the cerebral cortex of P3-P6 rats in the air-exposed control group, albeit these occurred relatively infrequently and had a smaller size. Approximately 24 hr after one (P4) or three (P6) days of ethanol vapor exposure, we detected a significant increase in the frequency and size of micro-hemorrhages in the surface of the cerebral cortex. Interestingly, this effect was not observed in brains collected at the end of the 3 hr ethanol vapor exposure at P3. We have previously shown that blood ethanol concentrations gradually decrease over the course of several hours after the end of exposure to a similar ethanol vapor chamber paradigm (Topper et al., 2015). Therefore, it is possible that the micro-hemorrhages could have started to develop at any time during the descending limb of the blood ethanol concentration curve. Alternatively, these could have developed during ethanol withdrawal. Ethanol exposure and/or withdrawal could have caused rupturing of developing blood vessels by increasing cerebral vascular resistance and/or systemic blood pressure (Altura et al., 1983, Kahkonen, 2004, Liu et al., 2004). In addition, alterations in blood-brain barrier integrity, secondary to neuronal injury, oxidative stress, activation of proteolytic enzymes, and/or neuroinflammation, could have contributed to the mechanism of micro-hemorrhage induction (Haorah et al., 2007, Yang and Rosenberg, 2011, Topper et al., 2015). Ethanol-induced inhibition of platelet aggregation could have also played a role in this process (Mikhailidis et al., 1983).

Other types of insults have been reported to induce brain micro-hemorrhages in the cerebral cortex of neonatal rats. Landolt and Arn (1979) found that exposure of P1-P2 rats to ionizing radiation induced petechial micro-hemorrhages in the cerebral cortex, which appear to have similar characteristics of those observed under our experimental conditions. Kotkoskie and Norton (1990) compared the effect of exposing pregnant rats to either ionizing radiation (gestational day 15) or ethanol (18 g/kg; gestational days 14 and 15; blood ethanol levels not determined). These investigators found that exposure to radiation causes widespread cell death in the fetal cerebral cortex at gestational day 16. In contrast, ethanol exposure caused both cortical thinning and micro-hemorrhages around the cerebral ventricles. This finding suggests that ethanol exposure during the rat equivalent to the second trimester of human pregnancy can also cause micro-hemorrhages in the fetal brain, although these are predominantly found at a different location than those caused by third trimester ethanol exposure. (Pahlavan et al., 2012) demonstrated that exposure of newborn rats to hypoxia (50-60% reduction in O₂ tension with respect to ambient air), administered in conjunction

with the vasoconstrictor agent, phenylephrine, induced petechial hemorrhages in several brain regions, some of which overlapped with those that showed micro-hemorrhages in our ethanol vapor exposure model (i.e., cerebral cortex, hypothalamus, midbrain, olfactory tubercle, striatum). The similarity between the patterns of micro-hemorrhages found in the brains of neonates exposed to hypoxia/vasoconstriction and ethanol is unlikely to be a consequence of the presence of low O₂ levels in the ethanol chambers, as these were minimally reduced to 92% of control in the high dose experiments. Moreover, we observed a similar increase in brain surface micro-hemorrhages in rats exposed to ethanol via intra-esophageal gavage. Therefore, the micro-hemorrhages are likely the result of direct actions of ethanol on the developing cerebral microvasculature. As discussed above, ethanol-induced cerebral vasoconstriction is one of the factors that could be involved in the mechanism of action of ethanol and this may explain the similarities between our results and those of Pahlavan et al., (2012).

It should be kept in mind that the stress associated with both the vapor and gavage ethanol exposure paradigms could have enhanced the effects of ethanol in the microvasculature, contributing to micro-hemorrhage generation. Consequently, it is important to determine if a less stressful ethanol exposure paradigm also causes micro-hemorrhages. The guinea pig animal model could be particularly useful in this context because these animals have a long gestation, with the brain growth spurt occurring *in utero* as in humans (Cudd, 2005). Moreover, a previous study showed that pregnant guinea pigs can be successfully exposed to moderate ethanol concentrations using a 24-hour voluntary drinking paradigm, which is expected to be less stressful (Shea et al., 2012). It is also important to determine whether ethanol exposure during the equivalent to the first and second trimesters of human pregnancy (i.e., pregnancy in rodents; Clancy et al., 2007) also causes fetal brain micro-hemorrhages and whether these contribute to the morphological and functional alterations that have been detected in different brain regions, including the cerebral cortex (Kuhn and Miller, 1998, Climent et al., 2002, Fakoya and Caxton-Martins, 2006, El Shawa et al., 2013, Abbott et al., 2016).

Ethanol-induced brain micro-hemorrhages were not observed in the brains of juvenile rats, suggesting that immature blood vessels are particularly susceptible to rupture secondary to exposure to this agent. During the first week of life, blood vessels in the rat brain are fragile because they are surrounded by a weak basement membrane and a large perivascular space (Caley and Maxwell, 1970, Krum et al., 1991). Moreover, the basement membrane is in a state of significant remodeling during this period of intense angiogenesis, being actively degraded by proteases released from migrating endothelial cells and axonal growth cones (Kalebic et al., 1983, Zhang et al., 2005). At the end of the first week of life, astrocyte end feet form, leading to thickening of the basal membrane and perivascular space contraction (Caley and Maxwell, 1970). These changes have been postulated to contribute to the reduced frequency of spontaneous micro-hemorrhages in the brains of neonatal rats after P7 (Pavlik and Mares, 1992) and they may also be, in part, responsible for the reduced susceptibility to ethanol-induced microvascular damage in the juvenile rats.

The ethanol-induced micro-hemorrhages caused neuronal loss, as indicated by a reduction in the number of NeuN positive nuclei. Neuronal death is likely a consequence, not only of

ischemia secondary to the micro-hemorrhage, but also secondary to exposure to contents of lysed blood cells as well as plasma components such as thrombin and plasmin which are known to trigger edema, inflammation, neurite retraction, and apoptosis, with the neonatal brain being particularly sensitive to these effects (Xue and Del Bigio, 2005). The neuronal death caused by the micro-hemorrhages was associated with a reactive astrocytic response around the area of the lesion. This finding is similar to that of Kenna et al., (2011) who detected areas of reactive gliosis around subarachnoid hemorrhages in brains of third trimester ethanol exposed fetal sheep. (Goodlett et al., 1993) also described focal areas of high intensity GFAP immunoreactivity in the cerebral cortex of P10 rats that were exposed to ethanol via artificial rearing between P4 and P9 (blood ethanol levels approximately 0.3 g/dl). Interestingly, these GFAP foci were located around small cortical blood vessels and it is possible that these were caused by micro-hemorrhages. Reactive astrogliosis could no longer be detected by P15 (Goodlett et al., 1993), which is consistent with the lack of focal areas of GFAP staining at P10 and later in our experiments. We found evidence consistent with microglia activation in the micro-hemorrhage area, suggesting that these cells played a role in clearance of cell debris and also repair of the damaged tissue (Neumann et al., 2009).

6. CONCLUSIONS

We describe here a novel mechanism by which ethanol exposure damages the developing brain. Importantly, the micro-hemorrhages were observed even at relatively moderate levels of ethanol exposure in the vapor chambers (i.e., near 0.08 g/dl, the legal intoxication limit in the U.S.). The National Birth Defects Prevention Study revealed that many women drink alcohol during the third trimester of pregnancy (Ethen et al., 2009). This behavior, in some cases, is reinforced by health care providers who are unaware of the potential dangers of drinking, even at low levels, during periods of gestation that are erroneously considered to be of lower susceptibility to ethanol-induced damage (Anderson et al., 2010, Sulik et al., 2012, Valenzuela et al., 2012). Our finding that moderate ethanol exposure during the third trimester equivalent can cause brain micro-hemorrhages adds to a significant body of scientific evidence from both animal and human studies indicating that it is not safe to drink any amount of alcohol during any period of gestation (Valenzuela et al., 2012, Flak et al., 2014, Williams and Smith, 2015). Postmortem studies have documented significant vascular alterations in the brains of human fetuses exposed to ethanol *in utero* (Jegou et al., 2012) and premature babies from women who reported relatively high ethanol consumption during pregnancy had an elevated risk for developing brain hemorrhages (Holzman et al., 1995). Therefore, the potential link between fetal ethanol exposure and cerebrovascular alterations should be further explored. Additional studies are also required to determine if the micro-hemorrhages are responsible for the behavioral and cognitive deficits observed in individuals with FASDs. Although the Catwalk test only revealed subtle deficits in locomotion and gait, it is possible that the rats were able to compensate for the damage caused by the third trimester-ethanol exposure-induced micro-hemorrhages or that this test lacks sufficient sensitivity to detect motor alterations secondary to the brain micro-hemorrhages. We previously found that ethanol vapor exposure for 4 hr/day during P2-P12 (peak pup blood ethanol levels near 0.3 g/dl) delayed the acquisition of the air-righting reflex, which was evident at P14-P18 (Diaz et al., 2014). Consequently, a more detailed assessment of

sensorimotor and cognitive functions in these animals is clearly warranted, including an in-depth characterization of these across different developmental time points.

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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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- Third trimester-equivalent ethanol exposure caused brain micro-hemorrhages
- This effect was observed both at low and high doses of ethanol exposure
- The micro-hemorrhages were located in the cerebral cortex and other brain regions
- The micro-hemorrhages caused neuronal loss and astrocyte/microglia activation

Figure 1. Ethanol vapor chamber exposure causes brain micro-hemorrhages

A) Schematic representation of the exposure paradigm. Pups, alongside dams, were exposed to air or ethanol in vapor chambers from 10 am to 1 pm on postnatal days (P) 3, 4 and/or 5. Samples were collected at the time points indicated in red. **B)** Exposure to high ethanol (EtOH) vapor levels resulting in blood ethanol concentrations of ~0.4 g/dl significantly ($P < 0.0001$) increased the average number of micro-hemorrhages in the surface of the brains at P4 ($n = 9-11$ pups from 3-4 litters/treatment group) and P6 ($n = 31-33$ pups from 8 litters/treatment group) but not at P3 ($n = 6$ pups from 2 litters/treatment group). **C)** Sample images of brains from air- and ethanol-(EtOH) exposed P6 rats illustrating the surface micro-hemorrhages. Sections outlined in red are enlarged on the right. Scale bar = 1.40 mm.

Figure 2. Further characterization of the brain surface micro-hemorrhages

A) Sample image of a brain from a postnatal day (P) 6 rat that was exposed to lower ethanol vapor levels resulting in blood ethanol concentrations near 0.08 g/dl. For a schematic illustration of the timing and duration of exposure, see Fig 1A. Scale bar = 2.29 mm. **B)** Exposure to lower ethanol (EtOH) vapor levels significantly increased the average number of micro-hemorrhages on the brain surface ($P < 0.0001$; $n = 22-26$ pups from 4 litters/treatment group). **C)** Sample image of a brain from a P6 rat that was exposed to ethanol on P3, P4, and P5 via intra-esophageal gavage resulting in blood ethanol concentrations near 0.4 g/dl. Scale bar = 2.29 mm. **D)** Exposure to high ethanol levels via gavage significantly increased the average number of micro-hemorrhages on the brain surface ($P < 0.0001$; $n = 15-14$ pups from 3 litters/treatment group). **E)** Sample image of a brain from a P24 rat that was exposed to ethanol vapor for 4 hr/day on P21, P22, and P23 resulting in blood ethanol concentrations near 0.3 g/dl. Scale bar = 1.81 mm. **F)** Exposure to high ethanol vapor levels for 4 hr/day for 3 days did not cause micro-hemorrhages on the brain surface of juvenile rats ($n = 34-38$ juvenile rats from 3 litters/treatment group).

Figure 3. The ethanol-induced micro-hemorrhages are associated with loss of neurons and reactive gliosis

A) Sample image of a cortical section from a postnatal day (P) 6 rat exposed to high ethanol levels, which were stained with antibodies that recognize neuronal nuclei (NeuN; scale bar = 100 μ m). For a schematic illustration of the timing and duration of exposure, see Fig 1A. The white rectangle represents the 50 μ m $\times$ 400 μ m area used to create the plot profile analysis of pixel intensity shown in **B**). The plot profile shows that the micro-hemorrhages were associated with a decrease in the intensity of immunolabeling for NeuN (shown is the mean plus 95% confidence interval of $n = 4$ brains from 4 litters). **C)** Same as in A but for sections stained with antibodies against the astrocytic marker glial fibrillary acidic protein (GFAP; green) and the nuclear marker 4',6-diamidino-2-phenylindole (DAPI; blue). **D)** The plot profile shows that the micro-hemorrhages were associated with an increase in the intensity of immunolabeling for GFAP (shown is the mean plus 95% confidence interval of $n = 4$ brains from 4 litters).

Figure 4. The ethanol-induced micro-hemorrhages are associated with microglia activation
A) Sample image of a cortical section from a postnatal day (P) 6 rat exposed to high ethanol levels, which were stained with antibodies that recognize the microglia marker, ionized calcium-binding adapter molecule 1 (IBA1; red) and the astrocytic marker glial fibrillary acidic protein (GFAP; green) (scale bar = 100 μm). For a schematic illustration of the timing and duration of exposure, see Fig 1A. **B)** Ethanol exposure (red bars) significantly increased the number of microglia with amoeboid and transitional morphologies within micro-hemorrhages, while reducing the number with resting morphology, with respect to air exposed controls (black bars) and cortical areas free of micro-hemorrhages in the ethanol exposed animals (orange bars) (for details on results of statistical analyses, please see text; $n = 3$ brains from 3 litters).

Figure 5. Effect of ethanol exposure to high ethanol levels on postnatal days (P) 3, P4 and P5 on motor function assessed in the Catwalk test at P48 and P71

Effect of ethanol on average body speed (A), step cadence (B), maximum paw contact area (C), step cycle (D), paw swing speed (E), and paw stride length (F). Right front paw (RF), right hind paw (RH), left front paw (LF), and left hind paw (LH). In all cases, $n = 15$ animals (from 7 air- and 8 ethanol-exposed litters). For details on results of statistical analyses, please see text. For additional Catwalk parameters, see Table 3.

Table 1

Ethanol increased brain micro-hemorrhages in a region-specific manner.

Brain Region	Control (n=10)	Ethanol (n=9)	Statistics ^a
Brain Stem	1.30 ± 0.49	2.55 ± 0.86	U = 29.5; P = 0.21
Cerebellum	2.90 ± 0.82	7.0 ± 1.91	t(17) = 2.04; P = 0.057
Cerebral Cortex	13.20 ± 2.68	68.33 ± 13.7	t(17) = 4.15; P = 0.0007***
Corpus Callosum	3.60 ± 0.88	2.33 ± 0.60	U = 33.5; P = 0.34
Hippocampus	0.70 ± 0.49	1.22 ± 0.4	U = 28.5; P = 0.15
Hypothalamus	0.0 ± 0.0	0.66 ± 0.33	U = 25; P = 0.03*
Midbrain	0.60 ± 0.26	2.88 ± 1.12	U = 18; P = 0.022*
Olfactory Tubercle	0.10 ± 0.1	2.55 ± 1.15	U = 17; P = 0.0079**
Striatum	0.20 ± 0.2	1.88 ± 0.73	U = 19.5; P = 0.015*
Thalamus	1.60 ± 0.71	4.77 ± 2.37	U = 26.5; P = 0.13
Ventricles	2.50 ± 0.76	2.11 ± 0.56	t(17) = 0.40; P = 0.69

^aMann-Whitney test was used to analyze data in rows where U values are provided.

Table 2

Ethanol increased micro-hemorrhages in selected regions of the cerebral cortex.

Cortical Region	Control (n=10)	Ethanol (n=9)	Statistics ^a
Auditory	0.0 ± 0.0	2.77 ± 0.83	U = 10; P = 0.007*
Cingulate	0.20 ± 0.13	1.0 ± 0.33	U = 22; P = 0.046*
Entorhinal	0.30 ± 0.21	0.44 ± 0.24	U = 39.5; P = 0.70
Frontal Association	0.60 ± 0.34	3.22 ± 1.16	U = 23.5; P = 0.058
Infralimbic	0.10 ± 0.10	0.0 ± 0.0	U = 40.5; P = 0.99
Insular	0.80 ± 0.38	3.88 ± 0.82	U = 7.5; P = 0.0008***
Motor	5.00 ± 1.18	15.22 ± 3.23	t(17) = 3.09; P = 0.0066**
Orbital	1.60 ± 0.42	6.66 ± 1.92	t(17) = 2.96; P = 0.015*
Parietal Association	0.20 ± 0.20	0.55 ± 0.29	U = 35; P = 0.37
Perirhinal	0.0 ± 0.0	0.11 ± 0.11	U = 40; P = 0.47
Piriform	0.10 ± 0.10	0.66 ± 0.37	U = 33.5; P = 0.19
Prelimbic	0.70 ± 0.30	1.66 ± 0.64	U = 31; P = 0.21
Retrosplenial	0.50 ± 0.30	3.88 ± 1.02	U = 7; P = 0.0006***
Somatosensory	2.60 ± 0.90	23.77 ± 6.82	U = 18; P = 0.025*
Subiculum	0.0 ± 0.0	0.33 ± 0.33	U = 40; P = 0.47
Visual	0.30 ± 0.15	3.33 ± 1.26	U = 16; P = 0.012*

^a Mann-Whitney test was used to analyze data in rows where U values are provided.

Table 3

Analysis of locomotion and gait in the Catwalk test.

Measure	Control	Ethanol	Statistics ^a
Swing (s)			
Left front paw	0.158 ± 0.018	0.179 ± 0.019	U=84; P=0.250
Left hind paw	0.157 ± 0.019	0.138 ± 0.005	U=111; P=0.967
Right front paw	0.198 ± 0.030	0.170 ± 0.011	U=107; P=0.838
Right hind paw	0.185 ± 0.043	0.140 ± 0.009	U=104; P=0.744
Relative Paw Placement			
Base of Support Front Paws (cm)	4.085 ± 0.145	3.828 ± 0.152	t(28)=1.238; P=0.22
Base of Support Hind Paws (cm)	5.661 ± 0.115	6.135 ± 0.234	t(28)=1.821; P=0.08
Support (%)			
Diagonal pairs	22.76 ± 3.512	31.73 ± 2.895	t(28)=1.971; P=0.06
Girdle pairs	1.463 ± 0.593	3.167 ± 1.795	U=108; P=0.867
Lateral pairs	7.606 ± 2.986	2.553 ± 0.597	U=91; P=0.389
Spatial Parameters			
Print Area (cm²)			
Left front paw	1.139 ± 0.114	0.916 ± 0.066	U=71; P=0.089
Left hind paw	1.174 ± 0.094	1.134 ± 0.132	U=95; P=0.486
Right front paw	1.098 ± 0.137	0.840 ± 0.038	U=83; P=0.233
Right hind paw	1.093 ± 0.089	0.980 ± 0.096	t(28)=0.868; P=0.39
Print Intensity (a.u.)			
Left front paw	91.81 ± 3.151	92.91 ± 2.023	t(28)=0.296; P=0.77
Left hind paw	93.42 ± 2.332	93.94 ± 2.672	t(28)=0.147; P=0.88
Right front paw	93.12 ± 2.818	92.89 ± 1.793	t(28)=0.066; P=0.94
Right hind paw	94.40 ± 2.451	93.42 ± 2.723	t(28)=0.268; P=0.79
Inter limb Coordination			
Phase Dispersions (%)			
Girdle pairs left hind paw (anchor) – right hind paw (target)	44.90 ± 1.996	42.68 ± 1.901	U=90; P=0.367
Diagonal pairs left front paw (anchor) – right hind paw (target)	18.07 ± 1.669	14.10 ± 1.279	t(28)=1.885; P=0.07
Diagonal pairs right front paw (anchor) – left hind paw (target)	21.33 ± 5.945	20.41 ± 6.218	U=81; P=0.202
Step Sequence (Number of Patterns)	13.60 ± 0.980	12.20 ± 0.428	U=90; P=0.351
Number of Steps	81.53 ± 5.136	68.07 ± 3.040	U=60.50; P=0.03*

^aMann-Whitney test was used to analyze data in rows where U values are provided (n = 15 animals/treatment group from 7 air- and 8 ethanol-exposed litters)