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In Utero Exposure to Alcohol Impairs Reactivity of Cerebral Arterioles and Increases Susceptibility of the Brain to Damage Following Ischemia/Reperfusion in Adulthood

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Abstract

Background: Maternal consumption of alcohol produces abnormalities in the developing fetus and can contribute to an increased incidence of many cardiovascular-related diseases. The first goal of this study was to determine whether *in utero* exposure to alcohol influences reactivity of cerebral arterioles in adult (12–15-weeks old) rats. The second goal of this study was to examine whether *in utero* exposure to alcohol increased the susceptibility of the brain to damage following an ischemic event in adult rats.

Methods: We fed Sprague-Dawley dams a liquid diet with or without alcohol (3% ethanol) for the duration of their pregnancy (21–23 days). In the first series of studies, we examined reactivity of cerebral arterioles to eNOS- (ADP), nNOS-dependent (NMDA) and NOS-independent agonists in adult rats before and during application of L-NMMA. In another series of studies, we examined infarct volume following middle cerebral artery occlusion in adult offspring exposed to alcohol *in utero*. In both series of studies, we also determined the role for an increase in oxidative stress by feeding dams apocynin for the duration of their pregnancy.

Results: We found that *in utero* exposure to alcohol reduced responses of cerebral arterioles to ADP and NMDA, but not to nitroglycerin in adult rats. In addition, treatment of the dams with apocynin prevented this impairment in cerebral vascular function. We also found that *in utero* exposure to alcohol worsened brain damage following ischemia/reperfusion in adult rats and that treatment of dams with apocynin prevented this increase in brain damage following ischemia/reperfusion.

Conclusions: We suggest that our findings may have important implications for the pathogenesis of brain abnormalities associated with fetal alcohol exposure.

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Author Contribution Statement

All authors contributed to the work presented in this paper. WGM conceived the study, SGC and WGM designed the experiments, performed the experiments, analyzed the data and wrote the manuscript.

Conflict of Interest

None.

Keywords

Fetal alcohol syndrome; nitric oxide; oxidative stress; cerebral vascular function; stroke

Introduction

Maternal consumption of alcohol during pregnancy is an established cause of fetal alcohol spectrum disorders (FASD) (May et al., 2018). Those affected by FASD experience an array of structural and developmental disorders of the brain and craniofacial regions, as well as abnormalities of the gastrointestinal, urinary, heart, liver, pancreas and vascular systems. In addition, those that have been exposed to alcohol *in utero* often suffer from cognitive decline, behavioral disorders, dementia, and seizures that manifest in early childhood and persist into adulthood (Daft et al., 1986; Coffin et al., 2005; Bell et al., 2010; Guerri et al., 2009). Studies that have applied the developmental origins of health and disease (DOHaD) approach reveal that adult-onset diseases (cardiovascular, diabetes, obesity, cognitive decline) appear to be programmed *in utero* in response to maternal exposure to many types of stimuli. Support for this concept can be found in studies suggesting that *in utero* exposure to a variety of agents and environmental stimuli can contribute to diseases in adulthood by targeting the endothelium and vascular function (Gray et al., 2015; Care et al., 2016; Jones et al., 2004; Sahna et al., 2000), suggesting mechanistic effects beyond toxicity-induced cell death. With regards to *in utero* exposure to alcohol, studies have shown a significant increase in cardiovascular abnormalities (atrial septal defects, ventricular septal defects and other malformations in blood vessels) in infants and children with FASD (Löser and Majewski, 1977; Jones et al., 1973; Davidson, 1989). However, there is a lack of information regarding the influence of *in utero* exposure to alcohol on the cerebral vasculature and on cerebral vascular diseases in humans. Although the precise mechanisms underlying intrauterine programming of adult diseases are not fully understood, they have been suggested to include alterations in the hypothalamo-pituitary-adrenal axis, cellular differentiation, gene expression and/or mitochondrial oxidative stress. In our previous study, we found that impaired responses of cerebral arterioles in adolescent rats (4–6 weeks old) exposed to alcohol *in utero* was related to an increase in oxidative stress (Cananzi and Mayhan, 2017). Unfortunately, there is a lack of information regarding the relationship between *in utero* exposure to alcohol and the prevalence of cerebral vascular disease in adulthood. Thus, the first goal of this study was to examine the influence of *in utero* exposure to alcohol in reactivity of cerebral arterioles in adult rats.

Ischemic stroke is a leading cause of mortality and long-term disability. While we and others have documented the effect of chronic alcohol consumption by adult animals on brain damage following cerebral ischemia/reperfusion (Ducroquet et al., 2013; Zhao et al., 2011; Zhao et al., 2010; Hillbom and Kaste, 1983), the effect of *in utero* exposure to alcohol on the susceptibility of the brain to ischemic injury during development has not been widely examined. One recent study (Bake et al., 2017) found that binge exposure of mice to alcohol (3 g/kg body weight twice daily) during GD12.5 through GD15.5 produced significant decrease in cranial-directed blood flow and a decrease capacity to compensate for brain injury (neurological deficits), but surprisingly these authors did not find an increase brain

infarct volume following an ischemic event at 3 months of age. Thus, very limited exposure to alcohol *in utero* did not appear to alter the susceptibility of the brain to damage following cerebral ischemia/reperfusion in young animals. The second goal of the present study was to determine the effect of *in utero* exposure to alcohol on brain damage after ischemia/reperfusion in adulthood. Given that reactive oxygen species (ROS) are a critical mediator of neuronal death and have been linked to neuronal damage during FASD and dysfunction following ischemia/reperfusion (Brocardo et al., 2011; Kalogeris et al., 2014; Navarro-Yepes et al., 2014; Cohen-Kerem and Koren, 2003), we also examined the role for an increase in oxidative stress in brain damage following cerebral ischemia in adult animals exposed to alcohol *in utero*.

Materials and Methods

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental diets.

We used virgin adult male (16-week-old) and female (16-week-old) Sprague-Dawley rats for breeding. One male and one female rat were allowed to mate overnight. Dams were then housed singly and assigned randomly to one of four groups that were fed one of the following liquid diets for the entire gestation period (21–23 days): Control (0% alcohol) diet, 3% alcohol diet, control+apocynin diet, or 3% alcohol+apocynin diet. Liquid diets were prepared daily, as we have described previously (Zhao et al., 2010; Mayhan, 1992b). The control diets contained 1.0 kcal/ml of which 35% are derived from fat, 47% from carbohydrates, and 18% from protein. The 3% alcohol diets contained 1.0 kcal/ml of which 35% are derived from fat, 18% from protein, 29% from carbohydrate and 18% from alcohol. The total daily volume of diet fed to the control animals was based upon the daily consumption of diet by the alcohol animals. The alcohol diet resulted in a blood alcohol concentration (BAC) of 13.6 ±1 mM at hour and 14.6±1 mM at 2 hours in the dams (Abcam ethanol assay kit (ab65343)). These values are equivalent to a BAC between 0.06% and 0.07%, respectively. BACs were only determined in the rats fed the alcohol alone diet and not the alcohol+apocynin diet. The liquid diets fed to the dams were replaced by a normal chow/water diet within a day of birth of the pups. Rats were weaned at 3 weeks of age and placed in cages with those of the same sex. Both male and female rats were used for the experiments. For the control+apocynin and alcohol+apocynin groups, we added apocynin (10 mg/kg/day) to the liquid diets fed to the dams for the duration of pregnancy.

Measurement of cerebral vascular reactivity.

The rats were prepared for studies at 12–15 weeks of age and were obtained from multiple different litters. On the day of the experiment, the rats were anesthetized with thiobutabarbital sodium (Inactin, 100 mg/kg IP) and a tracheotomy was performed. The rats were ventilated mechanically with room air and supplemental oxygen. A catheter was placed into a femoral vein for injection of supplemental anesthesia (10–30 mg/kg; as necessary). A

femoral artery was cannulated for the measurement of arterial blood pressure and to obtain a blood sample for the determination of blood gases.

A craniotomy was prepared over the left parietal cortex to visualize the microcirculation of the cerebrum. The cranial window was suffused with a bicarbonate buffer (2 ml/min) that was bubbled continuously with 95% nitrogen and 5% carbon dioxide. Temperature of the suffusate was maintained at 37 ± 1°C. The cranial window was connected via a three-way valve to an infusion pump, which allowed for infusion of agonists and antagonists into the suffusate. This method maintained a constant temperature, pH, pCO_2, and pO_2 of the suffusate during infusion of drugs. Arterial blood gases were monitored and maintained within normal limits throughout the experimental period. Diameter of cerebral arterioles was measured using a video image-shearing device. The cranial window was superfused with artificial cerebral spinal fluid for 30 min prior to testing responses of cerebral arterioles to the agonists.

Responses of cerebral arterioles were examined during superfusion of agonists that produce dilation dependent on the release of nitric oxide via activation of eNOS: ADP (10 and 100 μM); or nNOS: NMDA (30 and 100 μM). We also examined responses of cerebral arterioles to nitroglycerin (1.0 and 10 μM), which dilates cerebral arterioles independent of NOS. Diameter of arterioles was measured before, and at 1-minute intervals for 5 minutes during application of agonists. Baseline diameter of cerebral arterioles returned to control levels (before application of agonists) within 2–3 minutes after application of agonists was stopped. To determine the role of nitric oxide in ADP- and NMDA-induced dilation of cerebral arterioles, we suffused the cranial window with N^G-monomethyl-L-arginine (L-NMMA; 10 μM). L-NMMA has been shown to be a nonspecific inhibitor of all isoforms of NOS, and we and several others have used L-NMMA to examine the role of NOS in responses of cerebral arterioles to many agonists (Zheng et al., 2006; Mayhan, 1992a; Moncada and Higgs, 2006; Faraci and Heistad, 1991). Thus, after initially measuring responses of cerebral arterioles to the agonists, we started a continuous suffusion with L-NMMA. One hour after starting suffusion with L-NMMA, we retested responses of cerebral arterioles to the agonists.

Middle cerebral artery occlusion.

Cerebral ischemia was induced in rats by middle cerebral artery occlusion (MCAO) at 12–15 weeks of age. Rats were anesthetized with ketamine/xylazine (100/15 mg/kg IP, respectively). The left common and external carotid arteries were exposed and ligated. Occlusion of the middle cerebral artery (MCA) was accomplished through the insertion of a filament from the basal part of the external carotid artery and advancing it in the internal carotid artery to the point toward the location where the MCA branched from the circle of Willis. Occlusion of the MCA induced a rapid drop in blood flow to the cerebral hemisphere. Cerebral blood flow changes were assessed using a laser Doppler (Periflux System 5000, Perimed) flow probe attached to the parietal bone during the placement of the suture. The MCA was occluded for 90 minutes and reperfusion (24 hours) was initiated by removing the suture from the internal carotid artery. Once the suture was removed, the internal carotid artery was ligated.

At the end of the recovery period (24 hours after reperfusion), rats were neurologically assessed on a 24-point scale using a sensorimotor testing grade that measured spontaneous activity, symmetry of movement, response to vibrissae touch, floor walking, beam walking, symmetry of forelimbs, climbing, and reaction to touch (Garcia et al., 1995; Zhao et al., 2011). After the neurological evaluation, the rats were anesthetized and killed with an overdose of Inactin followed by a thoracotomy. The brains were removed, placed in ice-cold saline for 5 minutes and cut into six 2-mm coronal sections. Then, 2,3,5 triphenyltetrazolium chloride (TTC; 2%) was used to stain the brain sections. Images of the ischemic lesion were evaluated using NIH Imaging Software (ImageJ; version 1.52a). The percentage lesion was calculated by measuring the infarct damage area in the ipsilateral hemisphere and dividing by the total area of the contralateral hemisphere. Total, cortical, and subcortical lesions were evaluated.

Superoxide levels.

We measured superoxide anion levels using lucigenin-enhanced chemiluminescence. After transcardially perfusing the heart with cold normal saline, the brain was removed and immersed in a modified Krebs-HEPES buffer. Tissue samples from the parietal cortex were weighed and placed in polypropylene tubes containing 5 μM lucigenin. The samples were read in a Berthold Sirius luminometer, which reports relative light units emitted integrated over 30 second intervals for 5 minutes. Data were corrected for background and normalized to tissue weight. We measured superoxide levels under basal conditions and during exposure to NADPH (10 and 100 μM) for 5 minutes.

Statistical analysis.

A three-way ANOVA was used to compare findings between groups of rats (Figures 1, 5 and 6). Bonferroni correction was used to test for significance between groups. Alcohol, apocynin and sex were factors. No difference was observed between male and female rats, and thus data from males and females were pooled. There were significant interactions of alcohol and apocynin. Paired t tests were used to compare responses of arterioles to the agonists before and following application of L-NMMA (Figures 2, 3 and 4). Values are mean±SEM. A p-value of 0.05 or less was considered to be significant.

Results

Responses to the agonists.

Baseline diameter of cerebral arterioles, mean arterial pressure, and body weight were similar in all groups at 12–15 weeks of age (Table 1).

Application of ADP, NMDA, and nitroglycerin dilated cerebral arterioles in all groups of rats (Figure 1). However, vasodilation in response to ADP and NMDA, but not to nitroglycerin, was reduced in rats exposed to alcohol *in utero* (Figure 1). In addition, treatment of the dams with the control+apocynin diet did not influence responses of cerebral arterioles to the agonists when compared to responses of arterioles in control rats not exposed to apocynin *in utero* (Figure 1). Treatment of the dams with the alcohol+apocynin diet prevented the impairment in cerebral vasodilation in response to ADP ([Low dose

ADP=effect of ethanol: F(1, 52)=12.35, p<0.0009; effect of apocynin: F(1, 52)=14.06, p<0.0004; interaction of ethanol and apocynin: F(1, 52)=14.45, p<0.0004] and [High dose ADP=effect of ethanol: F(1, 52)=14.57, p<0.0004; effect of apocynin: F(1, 52)=31.30, 0.0001; interaction of ethanol and apocynin: F(1, 52)=46.57, p<0.0001]) and NMDA ([Low dose NMDA=effect of ethanol: F(1, 52)=10.65, p<0.0019; effect of apocynin: F(1, 52)=10.96, p<0.0017; interaction of ethanol and apocynin: F(1, 52)=10.76, p<0.0019] and [High dose NMDA=effect of ethanol: F(1, 52)=16.46, p<0.0002; effect of apocynin: F(1, 52)=10.00, p<0.0026; interaction of ethanol and apocynin: F(1, 52)=37.55, p<0.0001]) in 14–16-week-old adult rats (Figure 1). Thus, there were significant interactions of alcohol and apocynin.

Responses following L-NMMA.

Topical application of L-NMMA reduced baseline diameter in control, control+apocynin, and in alcohol+apocynin rats, but had no effect on baseline diameter of rats exposed to alcohol *in utero* (Table 2). In addition, L-NMMA reduced responses to ADP (Figure 2) and NMDA (Figure 3) in control, control+apocynin, and alcohol+apocynin rats, but had little effect in rats exposed to alcohol *in utero*. L-NMMA had no effect on responses of cerebral arterioles to nitroglycerin in any group (Figure 4).

Middle cerebral artery occlusion.

MCAO produced a similar decrease in cerebral blood flow in control (61±5% reduction), alcohol (49±6% reduction), control+apocynin (53±5% reduction) and alcohol+apocynin (48±7% reduction) rats (p>0.05). However, we found a significant increase in total infarct ([effect of ethanol: F(1, 46)=5.987, p<0.0183; effect of apocynin: F(1, 46)=10.96, p<0.0519; interaction of ethanol and apocynin: F(1, 46)=3.066, p<0.0866]) and subcortical infarct ([effect of ethanol: F(1, 46)=10.34, p<0.0183; effect of apocynin: F(1, 46)=4.979, p<0.0306; interaction of ethanol and apocynin: F(1, 46)=8.985, p<0.0044]) volumes in rats exposed to alcohol *in utero* compared to control, control+apocynin and alcohol+apocynin rats (Figure 5). In addition, neurological scores were lower in alcohol rats ([effect of ethanol: F(1, 46)=10.44, p<0.0023; effect of apocynin: F(1, 46)=4.685, p<0.0357; interaction of ethanol and apocynin: F(1, 46)=3.897, p<0.0544]) when compared to control, control+apocynin and alcohol+apocynin rats (Figure 5) indicating a worse outcome following ischemia/reperfusion in rats exposed to alcohol *in utero*.

Superoxide.

We found that superoxide levels from cortex tissue were increased in rats exposed to alcohol *in utero* under basal conditions ([effect of ethanol: F(1, 88)=14.20, p<0.0003; effect of apocynin: F(1, 88)=76.58, p<0.0001; Effect of ethanol and apocynin: F(1, 88)=21.92, p<0.0001]) when compared to the other groups of rats (Figure 6). In addition, this basal increase in superoxide levels observed in rats exposed to alcohol *in utero* was decreased in rats from dams that were fed the alcohol+apocynin diet. Stimulation of cortex tissue with NADPH, the substrate for NADPH oxidase, produced a significant increase in superoxide levels in rats exposed to alcohol *in utero* when compared to all other groups ([effect of ethanol: F(1, 88)=40.42, p<0.0001; effect of apocynin: F(1, 88)=18.91, p<0.0001; interaction of ethanol X apocynin: F(1, 88)=12.31, p<0.0007 for low dose] and ([effect of

ethanol: F(1, 88)=140.6, p<0.0001; effect of apocynin: F(1, 88)=121.8, p<0.0001; interaction of ethanol and apocynin: F(1, 88)=142.1, p<0.0001 for high dose]) (Figure 6).

Discussion

There are several new findings from this study. First, exposure to alcohol *in utero* impairs eNOS- and nNOS-dependent dilation of cerebral arterioles in adult (12–15-week-old rats). Second, brain infarct volume and neurological deficits following cerebral ischemia/reperfusion are increased in adult rats exposed to alcohol *in utero*. Third, *in utero* exposure to alcohol produced a sustained increase in basal levels of superoxide in the brain of adult rats and increased the ability of brain tissue to produce superoxide in response to NADPH (the substrate for NADPH oxidase). Fourth, treatment of the dams with apocynin to inhibit oxidative stress prevented *in utero*-induced impairment of cerebral vascular function, the increase in brain damage following cerebral ischemia/reperfusion and the basal increase in oxidative stress observed in adult rats. We suggest that our findings may have important implications for the pathogenesis of symptoms associated with FASD in adults, i.e., cognitive decline, behavioral disorders, dementia, and seizures (Daft et al., 1986; Coffin et al., 2005; Bell et al., 2010; Guerri et al., 2009), all of which may be influenced by the ability of the brain to maintain adequate cerebral blood flow in the face of changes in metabolic demand (neurovascular coupling).

We used ADP and NMDA as our eNOS- and nNOS-dependent agonists, respectively. We also used nitroglycerin as a NOS-independent agonist. Previous studies have shown that ADP binds to the $P2Y_{12}$ G protein-coupled receptor, NMDA opens NMDA receptors allowing the inward passage of Ca^{2+} ions, while nitroglycerin releases free nitric oxide following enzymatic action by dehydrogenases (Lewis et al., 2000; Garthwaite et al., 1989; Harrison and Bates, 1993). We and others have shown that ADP dilates cerebral arterioles through a pathway involving eNOS (Faraci, 1991; Mayhan, 1992a; You et al., 1997). NMDA has been shown to dilate cerebral arterioles through a pathway involving nNOS (Faraci and Breese, 1993; Busija and Leffler, 1989; Faraci and Brian, 1995). Thus, it appears that ADP and NMDA are appropriate agonists to examine eNOS- and nNOS-dependent dilation in cerebral arterioles.

In the present study we found that L-NMMA produced modest constriction of cerebral arterioles in control, control+apocynin and alcohol+apocynin rats, but not in alcohol-exposed rats. This finding suggests that basal production of nitric oxide contributes to baseline diameter of cerebral arterioles in all groups except the alcohol-exposed rats. In light of our finding showing that basal levels of superoxide are increase in rats exposed to alcohol *in utero*, we suggest that these basal levels of superoxide may be sufficient to impair the ability of nitric oxide to influence baseline diameter of cerebral arterioles.

The influence of sex on reactivity of peripheral arteries has been examined, but less is known about the influence of sex on reactivity of cerebral arterioles. In a previous study, we found that dilation of cerebral arterioles to ADP and NMDA was elevated in female compared to male rats (Arrick et al., 2016). In contrast, we did not find sex-related differences in reactivity of cerebral arterioles in the present study. The discrepancy between

the present study and our previous study is not clear, although the rats in the present study were older than in our previous study. One could suggest that feeding dams the liquid diets may have influenced reactivity to the agonists differently in male and female offspring, although this possibility seems unlikely. In any event, we are not certain as to why we did not see sex-related differences in vascular reactivity in the present study.

Several studies have examined the influence of *in utero* exposure to alcohol on reactivity of cerebral and peripheral blood vessels. Gleason *et al.,* (Gleason et al., 1997) and Mayock *et al.*, (Mayock et al., 2007) found that binge alcohol consumption in the second trimester impaired hypoxia-induced increases in cerebral blood flow in preterm and newborn (1–4 days old) sheep. However, underlying mechanisms were not examined. Likewise, Ngai *et al.*, (Ngai et al., 2008) reported that alcohol consumption by pregnant ewes impaired responses of large cerebral arteries to vasoactive intestinal polypeptide (VIP) in adult (10–13 months old) offspring, but again mechanisms were not examined. Bake *et al.*, (Bake et al., 2017) reported a decrease in blood acceleration (Doppler blood flow and pulse-wave recordings) through the carotid arteries of adult mice (3–12 months old) exposed to prenatal alcohol, but mechanisms were not examined. In recent studies, we found that *in vivo* responses of cerebral arterioles to eNOS- and nNOS-dependent agonists were impaired in young rats (4–6 weeks old) exposed to alcohol *in utero* (Cananzi and Mayhan, 2017). Finally, Turcotte *et al.,* (Turcotte et al., 2002) reported that prenatal exposure to ethanol (6.4%) decreased relaxation of the aorta to carbamylcholine in rats 25 weeks after birth. Thus, limited evidence suggests that *in utero* exposure to alcohol can alter responses of cerebral and peripheral arteries in young, adolescent and adult animals. However, precise mechanisms and specific consequences to the brain have not been fully examined.

In the present study, we found that *in utero* exposure to alcohol specifically impaired eNOS- and nNOS-dependent reactivity of cerebral arterioles in adult rats exposed to alcohol *in utero*. We also found that this specific impairment in cerebral vascular function could be prevented by treatment of the dams with apocynin. These results extend and complement findings from our previous study (Cananzi and Mayhan, 2017) and from others (Bake et al., 2017; Gleason et al., 1997; Mayock et al., 2007; Ngai et al., 2008) by examining reactivity of cerebral resistance arterioles, by examining response in adult animals and by examining potential mechanisms that account for impaired responses of cerebral arterioles by *in utero* exposure to alcohol.

While many studies have examined the effect of chronic alcohol consumption by adult animals on brain damage following cerebral ischemia/reperfusion (Ducroquet et al., 2013; Zhao et al., 2011; Zhao et al., 2010; Hillbom and Kaste, 1983), the effect of *in utero* exposure to alcohol on the susceptibility of the brain to ischemic injury during development remains uncertain. One recent study (Bake et al., 2017) found that binge exposure of mice to alcohol (3 g/kg body weight twice daily) during gestational days 12.5 through 15.5 produced significant decrease in carotid artery blood flow and a decrease capacity to compensate for brain injury (neurological deficits), but surprisingly there was no increase in brain infarct volume following an ischemic event in these mice at 3 months of age. In the present study we found that exposure to alcohol *in utero* during the entire gestation period produced an increase in brain infarct volume in 12–15-week-old rats following cerebral ischemia/

reperfusion. In addition, in agreement with the previous study (Bake et al., 2017), we also found an increase in neurological deficits in adult rats exposed to alcohol *in utero*. We also extend the findings of the previous study (Bake et al., 2017) by examining the influence of treatment of the dams with apocynin on brain infarct volume and neurological deficits in adult rats exposed to alcohol *in utero*. The discrepancy between the present study and the previous study (Bake et al., 2017) regarding the influence of *in utero* exposure to alcohol on brain infarct volume is not clear, but may be related to species differences, the degree of the reduction of cerebral blood flow during the occlusion, the time of exposure to alcohol and the timing of the exposure to alcohol during the development of the fetus.

A major source of superoxide is NADPH oxidase (Konior et al., 2014; Griendling et al., 2000). Activation of NADPH oxidase has been linked to brain damage following cerebral ischemia/reperfusion (Granger and Kvietys, 2015; Tang et al., 2007; Kahles and Brandes, 2012; Chen et al., 2009; Tang et al., 2008; Kusaka et al., 2004; Walder et al., 1997). NADPH oxidase is composed by two membrane bound proteins, gp91phox and p22phox, and three cytosolic proteins p67phox, p47phox, and p40phox. When activated, the cytosolic subunits reach the membrane and interact with membrane bound subunits, transferring electrons from NADPH to oxygen to form superoxide. We used apocynin to inhibit NOX-related proteins. Although the specificity of apocynin has been questioned in the past several years (Wind et al., 2010; Heumuller et al., 2008), others have suggested that apocynin prevents the formation of a working NADPH oxidase complex by inhibiting the association of subunit p47phox to the gp91phox/p22phox complex (Stolk et al., 1994). We found a decrease in eNOS- and nNOS-dependent responses of cerebral arterioles, an increase in superoxide levels and an increase in brain damage following ischemia/reperfusion in rats exposed to alcohol *in utero.* We also found that treatment of dams with apocynin reduced the magnitude of these abnormalities. This finding seems to suggest that an increase in oxidative stress, possibly via activation of NADPH oxidase, may contribute to cerebral vascular dysfunction in rats exposed to alcohol *in utero*.

In summary, we examined the effects of *in utero* exposure to alcohol on eNOS- and nNOS-dependent reactivity of cerebral arterioles, the susceptibility of the brain to damage following an ischemic event and superoxide levels in adult rats. We found that *in utero* exposure to alcohol impaired responses of cerebral arterioles to eNOS- and nNOS-dependent agonists and increased the susceptibility of the brain to damage following cerebral ischemia/reperfusion in adult rats. In addition, we found that treatment of dams with apocynin prevented impaired reactivity in cerebral arterioles and the increase in brain damage following cerebral ischemia/reperfusion in adult rats exposed to alcohol *in utero*, suggesting an important role for an increase in oxidative stress. We speculate that our findings may have important implications for those that suffer from symptoms associated with FASD, i.e., cognitive decline, behavioral disorders, dementia and seizures (Daft et al., 1986; Coffin et al., 2005; Bell et al., 2010; Guerri et al., 2009). Although there are no longitudinal studies that have examined the risk of stroke in individuals that suffer from FASD, there are studies that have shown that the prevalence of epileptic seizures is much higher in individuals affected by FASD compared to the general population (Bell et al., 2010). Given that there appears to be a relationship between seizures/epilepsy and the onset

of stroke (Zelano et al., 2017), we speculate that individuals with FASD could be at a greater risk for the pathogenesis of cerebral vascular disorders, perhaps including stroke.

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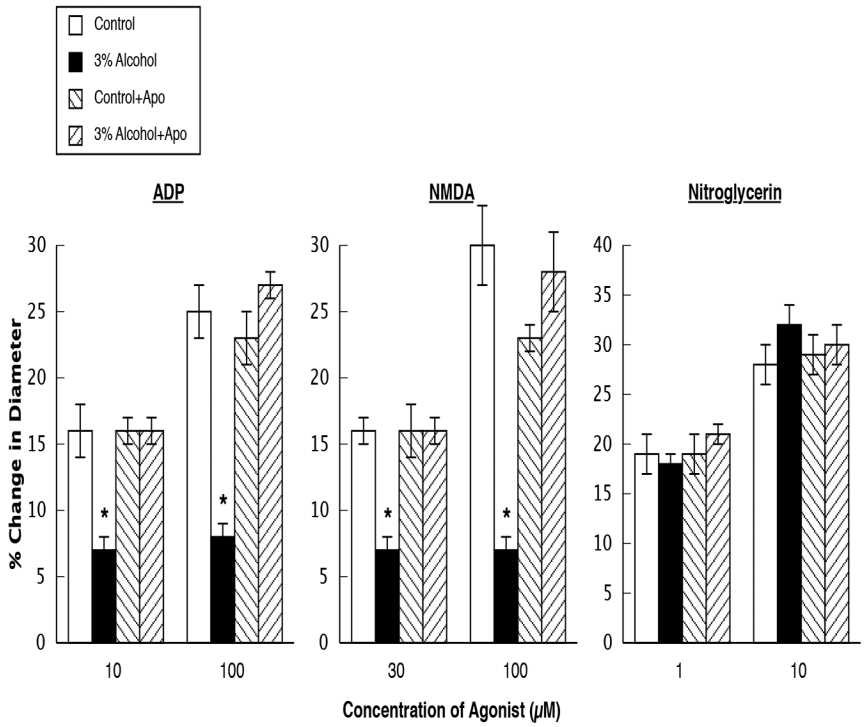

Figure 1.

Responses of cerebral arterioles to ADP, NMDA, and nitroglycerin in control rats, rats exposed to alcohol *in utero*, control+apocynin rats, and alcohol+apocynin rats. N=14 for each group of rats. Values are means ± SE. * $p<0.05$ versus control rats, control+apocynin rats and 3% alcohol+apocynin rats.

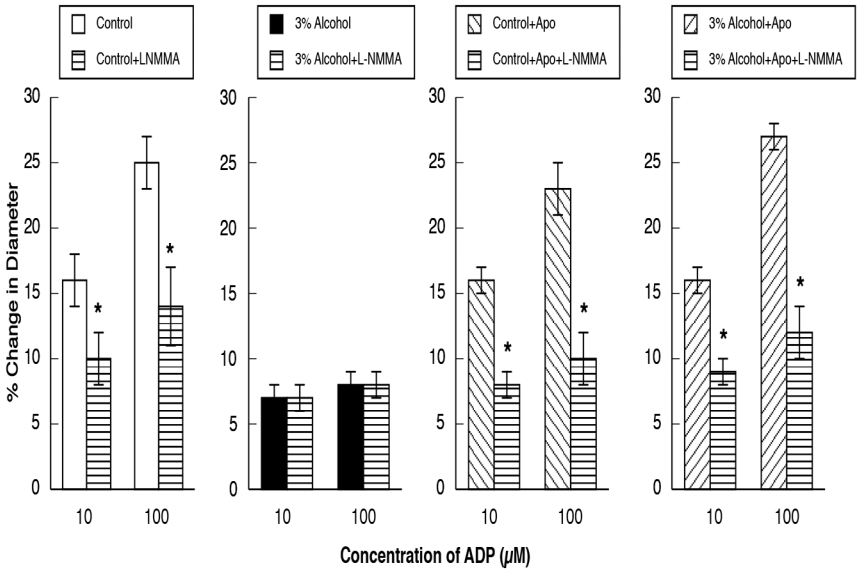

Figure 2.

Responses of cerebral arterioles to ADP in control rats, rats exposed to alcohol *in utero*, control+apocynin rats and alcohol+apocynin rats before and during suffusion of L-NMMA (10 μM). N=14 for each group of rats. Values are means ± SE. * $p<0.05$ versus response before L-NMMA.

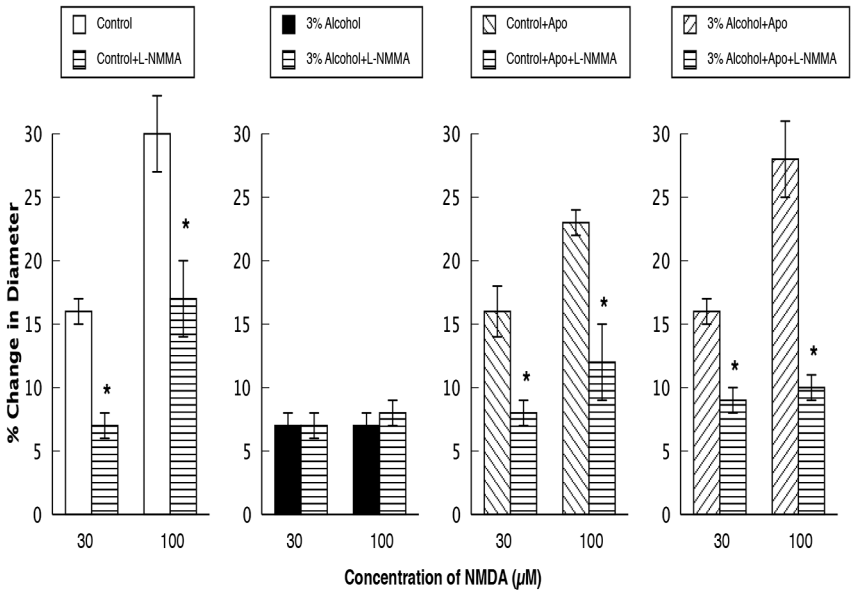

Figure 3.

Responses of cerebral arterioles to NMDA in control rats, rats exposed to alcohol *in utero*, control+apocynin rats and alcohol+apocynin rats before and during suffusion of L-NMMA (10 μM). N=14 for each group of rats. Values are means ± SE. * $p<0.05$ versus response before L-NMMA.

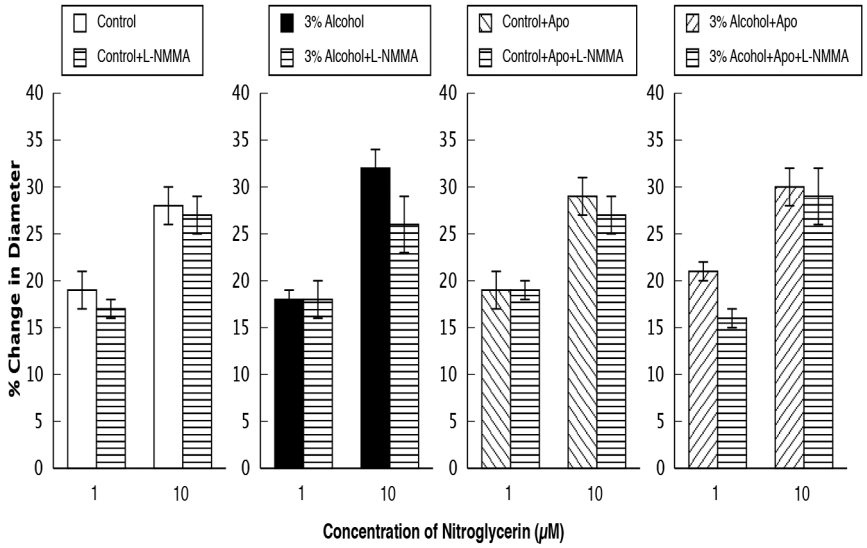

Figure 4.

Responses of cerebral arterioles to nitroglycerin in control rats, rats exposed to alcohol *in utero*, control+apocynin rats and alcohol+apocynin rats before and during suffusion of L-NMMA (10 μM). N=14 for each group of rats. Values are means ± SE. *

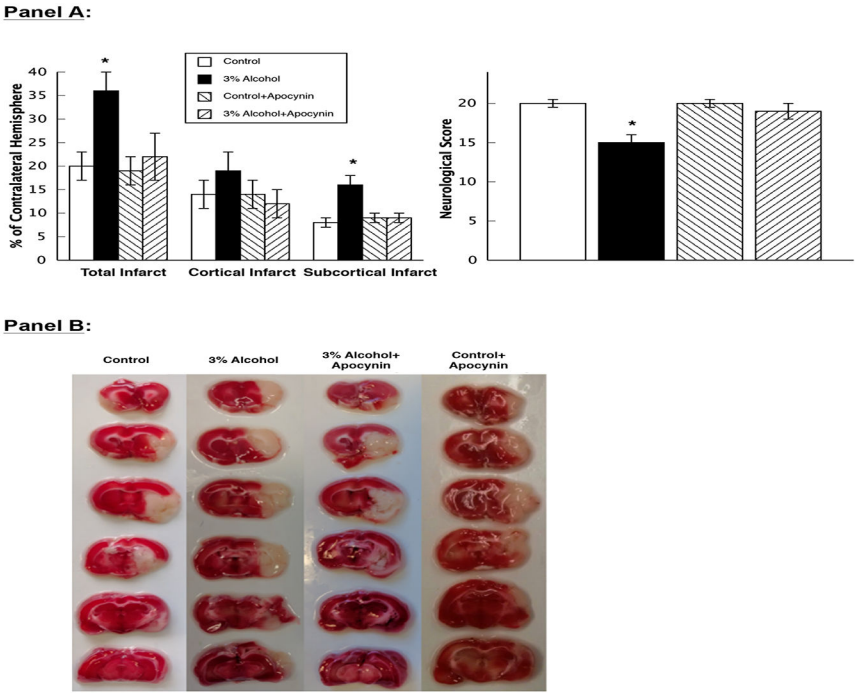

Figure 5.

Panel A: Mean data for total infarct, cortical infarct, and subcortical infarct volumes and neurological scores in control rats (n=13), rats exposed to alcohol *in utero* (n=12), control +apocynin rats (n=12), and alcohol+apocynin rats (n=13). Values are means ± SE. * $p<0.05$ versus control, control+apocynin and alcohol+apocynin rats. Panel B: Representative photograph of brain sections in control rats, 3% alcohol rats, control+apocynin rats and alcohol+apocynin rats after staining with TTC 24 hours following MCA occlusion.

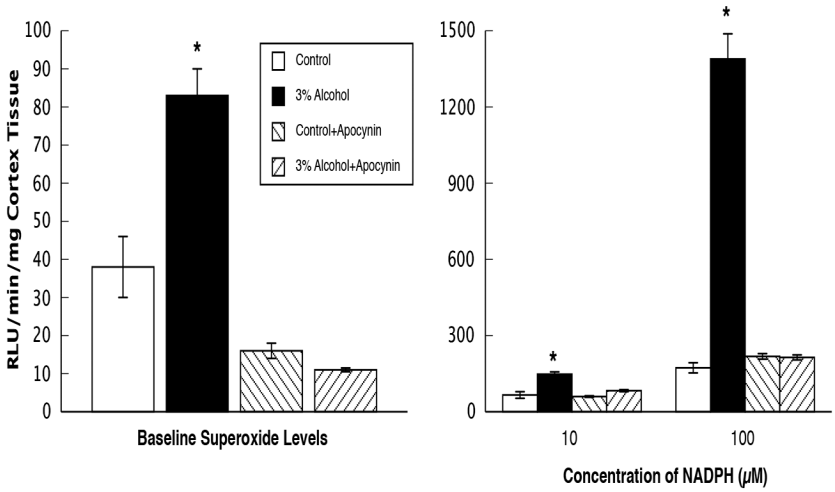

Figure 6.

Superoxide levels at baseline and during stimulation with NADPH (10 and 100 μM) in parietal cortex tissue from control rats, rats exposed to alcohol *in utero*, control+apocynin rats, and alcohol+apocynin rats. N=24 for each group of rats. Values are means ± SE.* $p<0.05$ versus responses in control, control+apocynin and alcohol+apocynin rats.

Table 1.

Baseline parameters at 14–16-week old rats.

	Control	Alcohol	Control+Apo	Alcohol+Apo
Baseline diameter (microns)	36±3	44±4	37±2	38±3
Mean arterial blood pressure (MAP) (mmHg)	114±4	110±3	108±2	114±2
Body weight (grams)	361±21	337±22	340±34	378±28

Values are mean±SE. n=14 for all groups of rats.

Table 2.

Baseline diameter of cerebral arterioles before and after application of L-NMMA.

	Before L-NMMA	After L-NMMA
Control rats	45±3	38±3 *
3% Alcohol rats	47±4	42±3
Control+Apocynin rats	46±3	36±2 *
3% Alcohol+Apocynin rats	49±4	36±3 *

All values are given in microns (μm). Values are means ± SE.

* $p<0.05$ versus diameter before application of L-NMMA (10 μM). n= 14 for all groups of rats.