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Mechanisms Underlying Chronic Binge Alcohol Exposure-Induced Uterine Artery Dysfunction in Pregnant Rat

Vishal D. Naik, M.S.¹, Katie Davis-Anderson, PhD¹, Kaviarasan Subramanian, PhD^{2,†}, Raine Lunde-Young, M.S.^{1,†}, Matthew J. Nemec, PhD¹, and Jayanth Ramadoss, PhD^{1,*}

¹Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX, 77843, USA

²Department of Obstetrics and Gynecology, University of Texas Medical Branch at Galveston, TX, 77555, USA

Abstract

Background—A cardinal feature of fetal alcohol syndrome is growth restriction. Maternal uterine artery adaptations to pregnancy correlate with birth weight and survival. We hypothesized that gestational binge alcohol exposure impairs maternal uterine vascular function, affecting endothelial nitric oxide-mediated vasodilation.

Methods—Pregnant rats grouped as pair-fed control or binge alcohol exposed received a once-daily, orogastric gavage of isocaloric maltose dextrin or alcohol, respectively. On gestational day 20, primary uterine arteries were isolated, cannulated, connected to a pressure transducer, and functional studies were conducted by dual-chamber arteriography. Uterine arteries maintained at constant intramural pressure (90 mm Hg) were maximally constricted with thromboxane, and a dose response for acetylcholine was recorded.

Results—The alcohol group exhibited significantly impaired endothelium-dependent, acetylcholine-induced uterine artery relaxation ($\downarrow \sim 30\%$). Subsequently, a dose response was recorded following inhibition of endothelium-derived hyperpolarizing factor (apamin and TRAM-34) and prostacyclin (indomethacin). Acetylcholine-induced relaxation in the pair-fed control decreased by $\sim 46\%$, and interestingly, relaxation in alcohol group further decreased by an additional $\sim 48\%$, demonstrating that gestational binge alcohol impairs the nitric oxide system in the primary uterine artery. An endothelium-independent SNP effect was not observed. Immunoblotting indicated that alcohol decreased the level of endothelial excitatory P-Ser¹¹⁷⁷eNOS ($P < 0.05$) and total eNOS expression ($P < 0.05$) compared to both the normal and pair-fed controls. P-Ser¹¹⁷⁷eNOS level was also confirmed by immunofluorescence imaging.

Conclusion—This is the first study to demonstrate maternal binge alcohol consumption during pregnancy disrupts uterine artery vascular function via impairment of the eNOS vasodilatory system.

Corresponding Author: Jayanth Ramadoss, PhD, Texas A&M University, Physiology and Pharmacology, 4466 TAMU, College Station, Texas 77843, United States, jramadoss@cvm.tamu.edu.

[†]Equal Contribution

DR. JAYANTH RAMADOSS (Orcid ID: 0000-0002-9778-5224)

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Introduction

Maternal alcohol exposure is strongly associated with a wide array of irreversible damage to the developing fetus, which can manifest as a range of physical and functional abnormalities, termed Fetal Alcohol Spectrum Disorder (FASD; Sokol et al., 2003). Recent reports estimate the prevalence of FASD to be between 2–5% in young school-aged children in both the United States and Western European countries (May et al., 2009). The most severe form of FASD, Fetal Alcohol Syndrome (FAS), was first defined as a pattern of altered growth, morphogenesis, and behavior in offspring of chronic alcoholic mothers (Jones and Smith, 1973). The cost burden of FAS in the United States was estimated to be \$6.5 billion in 2010 (Popova et al., 2011). Much of the emphasis within the FASD field remains directed towards understanding alcohol-mediated brain and behavioral deficits (Riley and McGee, 2005). Despite significant advances in discernment of neuropathogenesis, the mechanism(s) underlying gestational alcohol-mediated growth deficits remain essentially unknown. We reason that developmental deficits associated with gestational alcohol exposure may emanate from alcohol-induced alteration(s) to a mother's natural physiological adaptations associated with normal healthy pregnancy. Specifically, we focused the current study on alcohol-induced alteration(s) to maternal uterine vascular adaptations that occur during pregnancy.

During a normal pregnancy, dramatic adaptive changes to the maternal primary uterine artery ensure optimal nutrient and oxygen delivery for meeting the demands of fetal growth, development, and metabolism. In women, the primary uterine artery accomplishes this by markedly increasing its diameter compared to the non-pregnant state (1.4 mm to 3.4 mm by third trimester of pregnancy), which in turn corresponds to a striking decrease in uterine vascular resistance (Palmer et al., 1992, Magness et al., 1997). Alcohol has been reported to disrupt uterine arterial adaptations (Ramadoss and Magness, 2012), including blood flow in sheep (Falconer, 1990) and spiral artery remodeling in rats (Gundogan et al., 2008). In this study, we sought to examine the mechanism by which alcohol affects pregnancy-induced uterine vascular adaptation, specifically agonist-induced uterine vascular reactivity. Uterine artery vasodilation is mediated by three key endothelial-derived vasodilators: nitric oxide (NO), prostacyclin (PGI₂), and endothelial-derived hyperpolarizing factor (EDHF; Ramadoss and Magness, 2012). NO has been pivotally implicated in pregnancy, as prior works report both systemic and uterine artery-specific increases in NO production during pregnancy in humans (Williams et al., 1997, Nelson et al., 1998), and in various animal models such as rats (Conrad et al., 1993) and sheep (Magness, 1998). Interestingly, though a substantial body of work investigating *in vitro*, human, and animal models indicates that alcohol alters vascular NO production (Kay et al., 2000, Cook et al., 2001a), knowledge of this remains limited in the context of gestational alcohol exposure. One study has shown that alcohol decreased pregnancy-specific, NO-mediated vasodilation of the mesenteric artery in pregnant mice (Cook et al., 2001b). Though some investigation has explored the effect of gestational alcohol exposure on NO-mediated vasodilation in the systemic vasculature,

alcohol's impact on the reproductive vasculature, specifically the uterine artery, which primarily delivers oxygen and nutrients to the fetus, has yet to be established.

Endothelial nitric oxide synthase (eNOS) is enzymatically responsible for constitutive vascular NO production and its activity is regulated by multiple post-translational modifications (Qian and Fulton, 2013, Duran et al., 2010). One of the most essential and widely studied eNOS posttranslational modifications is the phosphorylation of serine at eNOS position 1177 (P- Ser¹¹⁷⁷eNOS), which directly leads to increased eNOS enzymatic activity (Mount et al., 2007). Previous *in vitro* investigations of uterine artery endothelial cells derived from pregnant sheep and human umbilical endothelial cells (Ramadoss et al., 2011, Subramanian et al., 2014a) have determined alcohol alters phosphorylation of Ser¹¹⁷⁷eNOS. Following the previous study by our group which determined alcohol induced vascular dysfunction of the uterine artery using wire myography (Subramanian et al., 2014), the current study assesses the mechanisms underlying *in vivo* alcohol exposure-induced uterine artery dysfunction in a pressurized vessel. We hypothesize that chronic binge alcohol exposure during pregnancy will decrease endothelial-dependent vascular relaxation in the maternal uterine artery by impairing NO-mediated vasodilation via alteration of eNOS post-translational modification.

Materials and Methods

Treatment groups and alcohol dosing paradigm

All experimental procedures were in accordance with National Institutes of Health guidelines (NIH Publication No. 85–23, revised 1996) with approval by the Animal Care and Use Committee at the Texas A&M University and University of Texas Medical Branch. Timed pregnant Sprague–Dawley rats purchased from Charles River (Wilmington, MA) were housed in a temperature-controlled room (23°C) with a 12:12-hour light–dark cycle. For dual chamber arteriograph experiments, two treatment groups were utilized, comprised of a nutritional pair-fed control group and a binge alcohol group. An additional untreated normal control group was included in immunoblotting studies (detailed in immunohistochemistry section). Dams in the alcohol treatment group acclimatized via a once-daily, binge-like, orogastric gavage dose of 4.5 g/kg ethanol (22.5% wt/v; peak BAC, 216 mg/dl) from gestational day (GD) 5–10, and progressed to a 6 g/kg alcohol dose from GD 11–20 (28.5% wt/v; peak BAC, 289 mg/dl) (Thomas et al., 2008, Thomas et al., 2010). To control for nutrition, daily feed intake of the alcohol rat was measured, and the pair-fed dams received an equal amount of feed as its weight- paired alcohol animal. Isocaloric maltose-dextrin was given once-daily to the pair-fed control rats to equate the calories derived from administered alcohol. The exposure regimen utilized in this study was modeled after reported alcohol consumption patterns in pregnant women and FASD animal models (Church and Gerkin, 1988, May et al., 2013, Thomas et al., 2008, Cudd et al., 2002).

Maternal, fetal, and placental weight measurements

Maternal weights of pregnant pair-fed control (n = 8) and alcohol (n = 8) rats were measured on GD 20, one day after the last ethanol administration on GD 19. Following euthanasia of

the dam, the concepti were carefully removed and each fetus and placenta were separated and weighed individually.

Endothelial-mediated vasodilation of the uterine artery

Functional uterine artery studies were conducted in a separate set of rats. Pregnant pair-fed control (n = 8) and alcohol-administered (n = 8) rats were utilized to study the functional response of the primary uterine artery. As previously described (Naik et al., 2016), following euthanasia, the whole uterus was transferred to a large 200 mm petri dish containing solidified Sylgard and filled with ice-cold HEPES-Bicarbonate Solution, pH 7.4 (NaCl 130 mM; KCl 4 mM; MgSO₄·7H₂O 2.5 mM; NaHCO₃ 4.05 mM; CaCl₂ 2.4 mM; HEPES 10 mM; KH₂PO₄ 1.18 mM; Glucose 6 mM; EDTA 0.024 mM), where it was pinned to facilitate uterine artery dissection. A segment of primary uterine artery (approximately 3–5 mm) was excised between bifurcations, trimmed of surrounding fat and connective tissue, and washed in HEPES- Bicarbonate Solution. Standard procedures for a dual-chamber arteriograph setup were utilized as described previously (Halpern et al., 1984). In brief, cleaned arterial segments from each group were mounted and secured via two nylon ligatures onto a glass cannula in a dual-vessel arteriograph chamber, which then was connected to a pressure transducer. Residual blood was removed from the vessel lumen and the free vessel end was ligated and securely fastened to the opposing cannula with nylon filament. Both chambers of the arteriograph were continuously circulated with HEPES buffer fixed at 37°C, and intraluminal pressure was maintained and monitored using a pressure servo controller and a pressure monitor, respectively. This setup advantageously permitted vessels from pair-fed control and alcohol-exposed dams to be studied consecutively, under uniform experimental conditions. Following cannulation and ligation, intraluminal pressure was increased to 60 mm Hg, at which pressure the vessel exhibited myogenic tone and then equilibrated for 1 hour (hr). After equilibration, intraluminal pressure was increased to 90 mm Hg to mimic *in vivo*-like conditions, at which pressure all data were recorded using Ion Wizard 6.6 (IonOptix) software. Vessels were pre-contracted with 10⁻⁷ M thromboxane (Tbx) for 20 minutes (mins). Tbx concentration for the current study was determined from the concentration response curve previously generated in our laboratory (Subramanian et al., 2014b). Tbx treatment was followed by administration of three-fold increasing doses of acetylcholine (Ach) from 10⁻¹⁰ M to 10⁻⁵ M, and the corresponding dose response was obtained. At each dose increment, data was recorded for 3–5 mins or until variable measurement remained constant.

Nitric oxide-mediated vasodilation of uterine artery

Following measurement of endothelium-mediated vasodilation, we examined the NO-mediated vascular relaxation. Endothelial cells produce NO by catalyzing L-arginine to L-citrulline. PGI₂ is produced by sequential oxidation of arachidonic acid by enzyme cyclooxygenases (COX-1 and COX-2) and elicits smooth muscle relaxation via cyclic AMP activation, whereas EDHF relaxes smooth muscle through hyperpolarization resulting from opening of Ca²⁺-activated K⁺ channels (Luksha et al., 2009). We selectively blocked the PGI₂- and EDHF-mediated vascular relaxation pathways in the primary uterine artery whilst preserving the intact NO pathway. Uterine arteries were washed in circulating fresh HEPES buffer for 15 mins followed by a 20 minute incubation in an inhibitor cocktail containing

indomethacin (10^{-5} M), a non-selective COX-1 and COX-2 inhibitor enzyme, and apamin (10^{-7} M) and TRAM-34 (10^{-7} M), which cooperatively inhibit Ca^{2+} -activated K^{+} channels. The vessel was again constricted by circulating 10^{-7} M Tbx, and vascular reactivity was recorded for increasing cumulative doses of Ach from 10^{-10} M to 10^{-5} M.

Immunoblotting

Since we were not limited to two treatment groups for this protocol as in dual chamber arteriography, an additional untreated normal control group was also utilized with the pair-fed control and alcohol treatment groups. The untreated normal control group received *ad libitum* food, the alcohol group received 6 g/kg ethanol as described in the alcohol-dosing paradigm, and the pair-fed groups received isocaloric maltose-dextrin, from GD 5–19, until the day of sacrifice. Following sacrifice on GD 20, uterine arteries were cleaned, dissected, flash frozen in liquid nitrogen, and stored at -80°C until the day of use. The arteries were homogenized and sonicated in 80–100 μl of ice cold RIPA buffer, pH 7.5 (220 mM Tris-HCl, 150 mM NaCl, 1 mM Na_2EDTA , 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM b-glycerophosphate, 1 mM Na_3VO_4 , 1 $\mu\text{g/ml}$ leupeptin; Cell Signaling Technologies #9806), and immediately placed on ice. Following centrifugation, the supernatant was carefully removed and stored in fresh 1.5 ml tubes. Protein concentration measurement was performed using Pierce™ BCA protein assay kit instructions (ThermoFisher Scientific). Twenty μg of protein from each sample was loaded onto a 4–20% tris-HEPES polyacrylamide gel (150V, 40 mins; Mini protein II, BioRad) alongside a full-range rainbow marker (GE Amersham). The proteins were then transferred onto a PVDF membrane, which was used for probing P-Ser¹¹⁷⁷eNOS (1:1000; #9571), β -Actin (1: 5000; #4967; Cell Signaling Technologies) as loading control, and total eNOS (1: 3000; #610297; BD Biosciences). Densitometry analysis was performed with AzureSpot software (Azure Biosystems).

Immunofluorescence

Maternal uterine arteries from random pregnant pair-fed control and alcohol rats were isolated on GD 21, flash frozen in Tissue-Tek Optimal Cutting Temperature compound (Sakura Finetek U.S.A., Inc.), and stored at -80°C until sectioning. 8 μm sections were cut using a Leica CM1860 cryostat (Leica Biosystems, Inc.), transferred onto positively charged microscope slides, and stored in a -80°C freezer until undergoing immunostaining. For staining, frozen sections were fixed consecutively with ice-cold methanol (30 min, -20°C) and 4% paraformaldehyde (30 minutes, 4°C), rinsed in phosphate-buffered saline (PBS), incubated in 10% normal serum (1hr), and incubated overnight (4°C) with either a primary antibody (1:200; P-Ser¹¹⁷⁷eNOS; Cell Signaling Technology; #9571 and 1:200; total eNOS, BD Biosciences; #610297) or PBS (negative control) in a humidified chamber. The next day, the tissues were incubated in goat anti-rabbit or goat anti-mouse secondary antibody (1:250; IgG Alexa 488; Invitrogen; #A11008 or #A11001, respectively) for 1hr at room temperature in the dark. After washing with PBS, 1 drop of anti-fade mounting media with DAPI (Invitrogen; #P36931) was applied to each slide, which were then coverslipped. Slides were again incubated overnight and were imaged the next day with a Zeiss Axioplan 4 fluorescent microscopy system using AxioVision 4.8 software (Carl Zeiss Microscopy GmbH).

Statistics

Maternal, fetal, and placental weights and placental efficiency were analyzed using a student's t-test. Vascular functional response data were analyzed using a two-way repeated measures ANOVA, with treatment group as the between factor and dose as the within factor, followed by analysis with Sidak's multiple comparison test. Immunoblotting data were analyzed utilizing one-way ANOVA, followed by *post hoc* analysis with Fisher's LSD. All data were analyzed using GraphPad Prism (GraphPad Software, Inc.) and are presented as mean $\pm$ SEM, with significance established *a priori* at $P < 0.05$.

Results

Maternal body weight was not different ($P = 0.1955$) between the pair-fed control and alcohol-fed rats (Figure 1). However, average fetal weight in the alcohol-administered rats (2.12 ± 0.07 g) was significantly lower ($P = 0.0015$) compared to that in the pair-fed control group (2.49 ± 0.06 g), an approximately 16% weight reduction. The observed fetal growth restriction depicts one of the hallmarks of FAS commonly used in diagnosis (Jones and Smith, 1973). Placental weight was not significantly different in alcohol-administered rats ($P = 0.2386$). Placental efficiency, calculated as fetal weight in grams divided by its respective placental weight in grams, which is an indirect measurement to calculate the efficiency by which placenta transfers nutrients to the fetus (Angiolini et al., 2006), was also not different between the two groups ($P = 0.1141$).

Alcohol significantly decreased uterine artery functional response in a pressurized vessel

Uterine arteries from the pair-fed control and alcohol-administered rats were mounted on the pressure arteriograph system and an Ach dose response was noted under identical conditions. A comparative illustration of dose-dependent vasodilatory response is depicted in Figure 2A. Maximal percent relaxation of the maternal uterine artery with increasing Ach dose administration was significantly lower in the alcohol group compared with that in the pair-fed control group (Figure 2B). At the highest Ach dose (10^{-5} M), uterine arteries of the alcohol-exposed rats exhibited ~30% decrease in endothelium-dependent vasodilation. This indicates a significant impairment of the uterine artery endothelial function following gestational alcohol exposure. In a separate set of pregnant pair-fed control ($n = 6$) and alcohol-administered ($n = 6$) rats, we observed no alcohol effect on endothelium-independent, sodium nitroprusside (SNP)-induced smooth muscle relaxation (Figure 2E).

Alcohol specifically altered NO-mediated uterine artery functional response

To distinguish the pathway(s) by which alcohol alters uterine artery vasodilation, vessels were incubated with EDHF and PGI_2 pathway inhibitors simultaneously. This treatment selectively preserved the NO pathway to remain intact as it has been established that blockade of all the three vasodilatory pathways with inhibitors by adding 10^{-4} M L-NAME (NO inhibitor) to the EDHF and PGI_2 inhibitor cocktail completely abolished the Ach-induced relaxation in the rat uterine artery (Chinnathambi et al., 2013). Following the incubation period, we assessed the vasodilatory response of these vessels to increasing cumulative Ach doses. As expected, uterine arteries from both the pair-fed control and

alcohol treatment groups exhibited a significantly reduced vasodilatory response after inhibition (Figure 2C, 2D). Mean vasodilation in the pair-fed control group decreased by ~46%. Interestingly, vasodilation in the alcohol-treated group decreased similarly by ~48%, which was in addition to the initial ~30% decrease prior to EDHF/PGI₂ inhibition. Together, these results suggest that the impaired vascular relaxation observed with gestational alcohol treatment was due to NO disruption.

Alcohol significantly altered Ser¹¹⁷⁷eNOS phosphorylation levels

Immunoblotting analysis—The phosphorylation level of excitatory Ser¹¹⁷⁷eNOS significantly decreased in uterine arteries of alcohol-administered rats compared to those of untreated normal as well as pair-fed controls (Figure 3). There was also a significant decrease in uterine artery total eNOS expression in the alcohol-administered rats compared with those in untreated normal and pair-fed control rats. Protein expression of β -actin, used as a loading control, was not different among treatment groups.

Immunohistochemical analysis—Uterine artery sections isolated from pair-fed control and alcohol rats were probed with a P-Ser¹¹⁷⁷eNOS antibody. Immunofluorescence imaging (Figure 4) demonstrated that eNOS was detected exclusively in the uterine artery endothelial cells. The level of fluorescence showed major decreases in the levels of phosphorylation at the Ser¹¹⁷⁷eNOS, thus complementing the immunoblotting analysis.

Discussion

This is the first study to demonstrate that *in vivo* binge alcohol exposure during pregnancy disrupts the endothelial NO-mediated vasodilation of the uterine artery. Collectively, three fundamental outcomes can be garnered from this study. During pregnancy, 1) alcohol significantly attenuates agonist-induced vascular reactivity in a rat uterine artery at physiological pressure of 90 mm Hg, 2) alcohol disrupts uterine artery vasodilation via the eNOS pathway, and 3) alcohol alters levels of excitatory post-translational modification and protein expression of eNOS in the rat maternal uterine artery.

FAS growth restriction is accompanied by maternal uterine artery dysfunction

In this study, pups from the alcohol-administered dams exhibited a significant decrease in fetal body weight when compared with pups from pair-fed control groups. Growth restriction is a hallmark phenotype of FAS, and our data align with previous studies performed using a similar exposure paradigm (Thomas et al., 2009, Sokol et al., 2003). Furthermore, our study is the first to show that maternal uterine artery dysfunction accompanies FAS-related growth restriction.

The current report corresponds with the previously reported observation that animal models of compromised pregnancies exhibit dysregulation of uterine vascular adaptations (Reynolds et al., 2006).

Gestational Alcohol Exposure Attenuates Vascular Reactivity

Chronic binge alcohol exposure during pregnancy attenuates uterine artery reactivity, a critical adaptation associated with pregnancy. During pregnancy, the maternal uterine vasculature undergoes extensive remodeling, characterized by a significant decrease in vascular resistance and a 30–50 fold increase in blood flow, ensuring adequate supply of oxygen and nutrient delivery to the developing fetus (Rosenfeld, 1977). These adaptations are in part associated with an increased endothelium-dependent vasodilation by three principal vasodilators, namely NO, PGI₂, and EDHF (Bird et al., 2003). Herein, we examined endothelial-mediated vasodilation of the late-gestation primary uterine artery by inducing vasodilation via increasing concentrations of Ach, which acts on the muscarinic receptors on the endothelial cells, thereby releasing these key vasodilators. Our results indicated that chronic, binge-pattern, gestational alcohol exposure significantly diminishes endothelium-dependent uterine artery vasodilation.

Impaired uterine artery vasodilation during pregnancy may increase resistance to blood flow and consequently diminish nutrient and oxygen delivery to the fetus, potentially contributing to deleterious and lasting health and developmental consequences. Many studies suggest increased oxidative stress to play a role in alcohol-induced systemic vascular dysfunction, although so far only *in vitro* studies have been performed on the uterine artery, thus warranting future investigations in this area. (Tufan and Satioglu-Tufan, 2003, Ramadoss and Magness, 2012). Our results align with prior studies, including studies on systemic (non-reproductive) vasculature, namely male rat tail artery, mesenteric artery, and aorta, and wire myography-based response in uterine artery (Subramanian et al., 2014b), all of which concluded that endothelium-dependent relaxation is blunted in response to alcohol exposure (Brizzolara et al., 1994, Hatake et al., 1989, Sahna et al., 2000, Hipolito et al., 2015).

Alcohol Disrupts the NO Pathway

Our data demonstrate that gestational alcohol disrupts the endothelial nitric oxide-mediated vasodilatory pathway in the primary uterine artery. In the absence of any inhibitors, alcohol decreased the maximal Ach-induced dilation by ~30%. Inhibition of the EDHF and PGI₂ pathways resulted in a significant, but most importantly, a further reduction of relaxation by ~46% in both the treatment groups. This indicates that the contributions of EDHF and PGI₂ to the uterine artery vasodilation in both the groups was of similar magnitude and not chiefly affected by alcohol. Thus, alcohol-induced dysregulation of Ach-induced vasodilation is mediated by impairment of NO component at least in the primary uterine artery.

Interestingly, a similar loss of NO-mediated, endothelium-dependent relaxation was also observed in myometrial arteries of women with pre-eclampsia (Kenny et al., 2002, Ashworth et al., 1997). Other studies have demonstrated chronic alcohol exposure decreases arterial and plasma NO levels (Passaglia et al., 2015, Husain et al., 2005, Husain et al., 2007) and NO-mediated relaxation in rat systemic vasculature (Mayhan, 1992, Sun and Mayhan, 2001, Sun and Mayhan, 2005), aligning with our observation that alcohol impairs endothelium-mediated uterine artery vasodilation by affecting NO system.

To examine if eNOS activity was affected in alcohol-fed rats, immunohistochemistry and immunoblotting experiments assessed maternal uterine artery excitatory Ser¹¹⁷⁷ eNOS

phosphorylation levels and eNOS expression. Decreased uterine vascular reactivity in alcohol-fed dams was concomitantly accompanied by a significant decrease in eNOS protein expression in uterine arteries compared to the untreated normal and pair-fed controls. Studies utilizing male rats have confirmed decreased renal (Sönmez et al., 2012) and aortic (Husain et al., 2008, Husain et al., 2007) eNOS levels following alcohol exposure. Prior studies utilizing isolated endothelial cell culture from uterine arteries of pregnant ewes (Ramadoss et al., 2011) and human umbilical vein endothelial cells (Subramanian et al., 2014a) ascertained that binge-like alcohol exposure decreased eNOS protein expression in these structures. Interestingly, immunoblot validation correspondingly revealed a significant decrease in the phosphorylation at the excitatory Ser¹¹⁷⁷eNOS. Phosphorylation of the Akt-dependent Ser¹¹⁷⁷eNOS is extensively associated with increased eNOS-mediated NO production in systemic vasculature (Dimmeler et al., 1999).

Mutation at the Ser¹¹⁷⁷ eNOS to mimic decreased phosphorylation attenuated NO release in various *in vitro* models (Bauer et al., 2003, Du et al., 2001). Further, *in-vivo* studies in rats have also found a direct relation between decreases in Ser¹¹⁷⁷eNOS phosphorylation with decreased NO production (Murata et al., 2002, Musicki et al., 2005, Soucy et al., 2006).

In summary, this is the first study to demonstrate maternal binge alcohol consumption during pregnancy impairs uterine artery vascular reactivity, specifically impeding eNOS pathway-mediated vasodilation. Diminished vasodilation of the uterine artery can have deleterious effects on healthy fetal development. Substantial evidence robustly links low birthweight infants with an increased risk of infant mortality and a heightened susceptibility for developing chronic diseases in later life, which experts recognize globally as the *fetal origin hypothesis* (Simmons et al., 2001, Barker, 1992). Thus, growth deficits incurred in response to alcohol exposure *in utero* may have life-long health consequences that are potentially costly and can negatively influence both lifespan and quality of life. Although it cannot be said with certainty whether the decreased vasodilation would be directly responsible for the observed fetal growth restriction, this study provides a possible connection between the maternal uterine artery eNOS pathway and FASD pathogenesis. Future studies are warranted to ascertain specifically how these disruptions of uterine vascular adaptations to pregnancy may negatively impact fetal growth and development.

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Figure 1. Effect of chronic binge alcohol exposure on fetal development

Maternal weight, fetal weight, and placental weight were measured on GD 20, one day after last alcohol exposure. Prenatal alcohol exposure A) did not affect mean maternal weight ($P = 0.1955$), however it significantly decreased B) mean fetal body weight ($P = 0.0015$). C) Mean placenta weight and D) placental efficiency (the ratio of fetal to placental weight) was not different between groups ($P = 0.2386$ and $P = 0.1141$, respectively).

Figure 2. Binge alcohol exposure during pregnancy attenuates NO-mediated vasodilation of the uterine artery in pregnant rats

A) Representative pressure arteriograph tracing showing a dose-dependent vasodilatory response (uterine artery diameter, Y-axis) of the pregnant rat uterine artery with cumulative increasing doses of Ach (X-axis). B) Endothelial-dependent vasodilation to Ach in uterine artery from pair-fed control (▲), and alcohol-administered rats (■). To exclusively study the NO-mediated vasodilation of the rat uterine artery, EDHF and PGI_2 pathways were blocked while the NO pathway was kept intact. C) Relaxation of the uterine artery isolated from pair-fed rats with (Δ) and without (▲) inhibitors, and D) relaxation of the uterine artery isolated from the alcohol-administered rats with (□) and without (■) inhibitors. E) Endothelium-independent vasodilation to SNP in uterine artery from pair-fed control (▲), and alcohol-administered rats (■). Data are expressed as mean $\pm$ SEM, of percent relaxation from maximal constricted state at 90 mm Hg.

Figure 3. Analysis of Total eNOS expression and phosphorylation level at Ser¹¹⁷⁷ eNOS by immunoblotting

Uterine artery from GD 20 pregnant rats were homogenized and total eNOS protein expression and level of P-Ser¹¹⁷⁷ eNOS were analyzed by immunoblotting. Data are expressed as mean $\pm$ SEM and as fold of β -actin (loading control). Significance was established *at priori* at $P < 0.05$.

Figure 4. Immunohistochemistry (IHC) analysis of P-Ser¹¹⁷⁷eNOS

Immunofluorescence staining shows a localized expression of P-Ser¹¹⁷⁷eNOS in the endothelium and a major decrease of excitatory P-Ser¹¹⁷⁷eNOS expression in the uterine artery of the alcohol-administered rat compared to expression in the pair-fed control.