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Maternal alcohol consumption in pregnancy enhances arterial stiffness and alters vasodilator function that varies between vascular beds in fetal sheep

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Key points

- To date, the effects of maternal alcohol consumption in pregnancy have focused on neuro-developmental outcomes, with the impact on the arterial system poorly understood.
- In this study we investigated the effects of moderate maternal alcohol consumption (~3 standard drinks per day) in late pregnancy on the ability of arteries in the fetus to relax, and hence deliver blood, and on arterial stiffness in five important organs.
- Maternal alcohol consumption in late pregnancy resulted in a marked increase in stiffness in arteries of the heart, kidney, gut, leg muscle and brain in the fetus, and this could slow contraction and relaxation and overall blood delivery.
- Coronary artery endothelial vasodilator function was severely blunted as a result of alcohol exposure, while in contrast endothelium-dependent relaxation in renal and mesenteric arteries was enhanced.
- Together, the results of this study provide a warning for pregnant women and their carers that maternal consumption of ~3 standard drinks per day, levels that do not evoke physical abnormalities or growth restriction, can dramatically alter arterial function in the fetus.

Abstract While the impact of alcohol consumption by pregnant women on fetal neuro-development has received much attention, the effects on the cardiovascular system are not well understood. We hypothesised that repeated exposure to alcohol (ethanol) *in utero* would alter fetal arterial reactivity and wall stiffness, key mechanisms leading to cardiovascular disease in adulthood. Ethanol (0.75 g (kg body weight)$^{-1}$) was infused intravenously into ewes over 1 h daily for 39 days in late pregnancy (days 95–133 of pregnancy, term ~147 days). Maternal and fetal plasma ethanol concentrations at the end of the hour were ~115 mg dl^{-1}, and then declined to apparent zero over 8 h. At necropsy (day 134), fetal body weight and fetal brain–body weight ratio were not affected by alcohol infusion. Small arteries (250–300 μm outside diameter) from coronary, renal, mesenteric, femoral (psoas) and cerebral beds were isolated. Endothelium-dependent vasodilatation sensitivity was reduced 10-fold in coronary resistance arteries, associated with a reduction in *endothelial nitric oxide synthase* mRNA ($P = 0.008$). Conversely, vasodilatation sensitivity was enhanced 10-fold in mesenteric and renal resistance arteries. Arterial stiffness was markedly increased ($P = 0.0001$) in all five vascular beds associated

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with an increase in elastic modulus and, in cerebral vessels, with an increase in *collagen Iα* mRNA. Thus, we show for the first time that fetal arteries undergo marked and regionally variable adaptations as a consequence of repeated alcohol exposure. These alcohol-induced vascular effects occurred in the apparent absence of fetal physical abnormalities or fetal growth restriction.

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Abbreviations 5-HT, 5-hydroxytryptamine; ACh, acetylcholine; alcohol, ethanol; AUC, area-under-curve; BK, bradykinin; COX, cyclooxygenase; EDHF, endothelium-derived hyperpolarising factor; ETR, endothelin receptor; FAS, fetal alcohol syndrome; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NOS, nitric oxide synthase; PE, phenylephrine; PG, prostaglandin; SNP, sodium nitroprusside.

Introduction

Alcohol use by pregnant women is 10–20% according to the 2010 US Centers for Disease Control and Prevention report (Prevention, 2010), and in Australia, 30–60% of women reported consuming one to seven alcoholic drinks per week in the 2nd and 3rd trimesters of pregnancy (Colvin 2007; Morley *et al.* 2010). There is unimpeded distribution of alcohol (ethanol) across the placenta, and the maternal and fetal blood ethanol concentration–time curves are virtually identical (Brien *et al.* 1985; Cudd *et al.* 2001). It is evident that heavy episodic (binge) drinking can significantly impact maternal endocrine and cardiovascular systems (Ramadoss & Magness, 2012). Thus, as a result of maternal alcohol consumption, the fetus is subjected both to the direct effects of alcohol and the consequences of changes in maternal physiology.

A well-studied consequence of substantial maternal alcohol consumption, called fetal alcohol syndrome (FAS), includes a characteristic facial dysmorphology, growth restriction and neurodevelopmental delay resulting in intellectual impairment (Pruett *et al.* 2013). In contrast to the major focus on neurological outcomes, the impact of fetal alcohol exposure during pregnancy on the cardiovascular system has received scant attention. It is well established that alcohol consumption in adults is causally linked with cardiovascular abnormalities (Kurihara *et al.* 2004; Fatjo *et al.* 2007; Fenelon *et al.* 2007; Djousse *et al.* 2009; Klatsky, 2009). Long-term alcohol consumption in adult rats induces dilated cardiomyopathy (Piano *et al.* 2007). The incidence of cardiac malformations in the offspring is as high as 20% consequent to heavy maternal alcohol consumption in early pregnancy, compared with 1.2% in control children (Tikkanen & Heinonen, 1991; Kvigne *et al.* 2004). Even moderate alcohol consumption is associated with a lower Apgar score at birth (Iveli *et al.* 2007), and maternal alcohol consumption at any stage in pregnancy resulted in a halving of the capacity of the umbilical cord to contract (Iveli *et al.* 2007). While arterial pressure was normal in 9-year-old children of women who had consumed alcohol in pregnancy, pulse-wave velocity was increased, suggesting enhanced vascular stiffness (Morley *et al.* 2010).

In experimental models of FAS, the incidence of kidney defects, urethral obstruction, renal hypoplasia and hydronephrosis is increased (Assadi, 1990; Taylor *et al.* 1994). Even with more modest alcohol exposure, nephron endowment is reduced in fetal sheep (Gray *et al.* 2008) and in rat offspring as adults (Gray *et al.* 2010). *In utero* alcohol exposure led to increased arterial pressure in adult rats (Turcotte *et al.* 2002; Gray *et al.* 2010), but it was without effect on blood pressure in sheep fetuses (Gray *et al.* 2008). In another study of sheep fetuses, cerebral blood flow was enhanced, but only in the cerebellum (Parnell *et al.* 2007) and the vasodilator response to hypoxia was reduced (Gleason *et al.* 1997) following alcohol exposure *in utero*. Endothelium-dependent relaxation in aorta was reduced in 25-week-old rat offspring from alcohol-affected pregnancies (Turcotte *et al.* 2002). These results suggest that the impact of prenatal alcohol exposure varies between vascular beds.

Owing to the limited understanding of the effects of prenatal alcohol exposure on the developing vasculature, our objective was to determine the effects of maternal alcohol consumption on endothelial vasodilator function and arterial stiffness in the fetus; these are both key vascular variables that, when perturbed, are an important basis for cardiovascular disease. The levels attained here, ∼3 standard drinks per day, approximate those measured in young women during social drinking (Moore *et al.* 2007). We focused on resistance arteries and determined the effects of alcohol exposure on mechanisms of arterial contraction and endothelium-dependent and -independent relaxation in major vascular beds in fetal sheep, namely, the coronary, renal, mesenteric, skeletal muscle (psoas), and cerebral circulations, at a time of peak structural and functional maturation of these organs.

Methods

Animals

All procedures were completed at Monash University and were approved by Monash University School of Biomedical Sciences Animal Ethics Committee A and were in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. On day 92 of pregnancy (term ~147 days), twelve Border-Leicester × Merino crossbred ewes, each carrying a single fetus, underwent isoflurane anaesthesia (Bubb *et al.* 2007) and catheters were implanted chronically into a jugular vein and carotid artery of the ewe. Starting on day 95 of pregnancy, ethanol was infused into the jugular vein at 0.75 g (kg body weight^{-1}) for 1 h (09.00 h to 10.00 h) per day to six of the ewes. The infused solution consisted of four parts absolute ethanol diluted with six parts of isotonic saline. Six control ewes received an equivalent volume of saline (~120 ml in a 50 kg sheep) over 1 h. Ewes were weighed every 2 weeks to adjust the alcohol dose for body weight gain. Food intake was monitored and control ewes were given extra Lucerne chaff to provide a total of 20 kJ per day to compensate for the additional calories contained in the alcohol dose that was infused.

Alcohol was not administered on day 125, and on day 126 each ewe was again anaesthetised, the fetal head and a forelimb were exposed, and a catheter was implanted into a brachial artery for blood sampling and recording of arterial pressure. Another catheter was placed in the amniotic cavity to measure amniotic fluid pressure. The ewes were allowed one day to recover and daily alcohol infusion was resumed on day 128 until day 133 and necropsy was performed on day 134. Heart rate and blood pressure were measured from days 131–133, using the analysis program Lab Chart 6 ADInstruments, Bellvista, NSW).

For the purpose of determining the effects of short-term alcohol exposure, an additional three ewes underwent surgery on day 126 and were infused with alcohol on days 131–133 of pregnancy, with necropsy on day 134.

Blood vessel function testing

On day 134 of pregnancy, 24 h after the last alcohol infusion, each ewe was killed with pentobarbitone (Lethabarb, 120 mg kg^{-1}), the fetus was removed, weighed and measured, and the fetal heart, brain, kidneys, psoas muscle and small intestines were removed and placed in ice-cold physiological saline solution (PSS) containing (in mM): 120 NaCl, 5 KCl, 1.2 $MgSO_4$, 25 $NaHCO_3$, 1 KH_2PO_4, 2.5 $CaCl_2$, 11 glucose, gassed with 95% O_2–5%CO_2 (Carbogen). Arteries were cleared of surrounding tissue and fat, segments were taken for wire and pressure myography, and the remaining arteries were snap frozen in liquid nitrogen for later molecular analysis.

Segments of arteries (250–300 μm outside diameter and 1–2 mm in length) were mounted on a 4-channel wire myograph for isometric tension recording as described previously (Bubb *et al.* 2007; Mazzuca *et al.* 2010). The segments were bathed with PSS at 36°C and smooth muscle and endothelial integrity were tested using the agent that provided the greatest contractile response and also provided optimal endothelial stimulation, as determined for each vessel type in preliminary studies. These were: the thromboxane mimetic U46619 to constrict the coronary artery, phenylephrine (PE) for the mesenteric and renal arteries, and 5-hydroxytryptamine (5-HT) for the cerebral and femoral (from the psoas muscle) arteries. Smooth muscle contractile function was tested using cumulative concentrations of these constrictor agents. Following 30 min rest, the arterial segment was sub-maximally constricted (~60% of maximal) and endothelial vasodilator release was induced by cumulative addition of the endothelial agonist bradykinin (BK) in all arteries except the femoral, for which acetylcholine (ACh) was used. Following 30 min rest, endothelium-derived nitric oxide (NO) synthase was inhibited by including N^{ω}-nitro-L-arginine methyl ester (L-NAME, 2×10^{-4} M) in the PSS. The process was repeated in the presence of indomethacin (2×10^{-6} M) to block cyclooxygenase (COX) and hence prostanoid (PG) production. Arterial relaxation persisting in the presence of L-NAME and indomethacin was attributed to endothelium-derived hyperpolarising factor (EDHF; Bubb *et al.* 2007). Endothelium-independent smooth muscle relaxation was tested using the NO donor sodium nitroprusside (SNP; Mazzuca *et al.* 2010). At the end of each experiment the blood vessel was exposed to high-K^+ PSS (isotonic replacement of NaCl with 100 mM KCl) to elicit a standard contraction.

Passive mechanical wall properties – arterial stiffness

Arterial stiffness was tested by mounting leak-free segments of artery in a pressure myograph (Living Systems Instrumentation, Burlington, VT, USA) with no luminal flow (Wigg *et al.* 2004). The segments were continuously superfused at 15 ml min^{-1} with PSS containing 2 mM EGTA but no added calcium. Pressure was increased in 10 mmHg increments from 0 to 120 mmHg and changes in outside diameter and wall thickness in response to the pressure increases were measured.

Gene expression analysis

Fetal coronary, mesenteric, renal, femoral and cerebral arterial tissue that was frozen at −80°C at necropsy was used for RNA extraction using an RNeasy mini kit (Qiagen, Australia), and relative mRNA levels of the following genes were measured using quantitative real-time PCR (qPCR): *collagens Ia1*, *Ia2* and *III, elastase*

Table 1. qPCR primers

Gene		Primer sequence	Primer concentration	Annealing temperature
Collagen I α1	Forward	AAGACATCCCACCAGTCACC	10 μM	60°C
	Reverse	CAGATCACGTCATCGCACA		
Collagen I α2	Forward	GGCTCAACCTGAAGACATCC	4 μM	59°C
	Reverse	TCTCCTACCCAGACATGCTTC		
Collagen III	Forward	CTGCTGGAAAGAATGGTGAG	10 μM	59°C
	Reverse	GTCACCAGAAGGCCCAGTA		
Elastase 2	Forward	AGACTCCTTTGCCTCTGTCG	10 μM	58°C
	Reverse	AGCCTTTCTAGTGGGTCCTG		
Tropoelastin	Forward	ATCTCTCAGTCAGGCACCAG	10 μM	58°C
	Reverse	GTTTGTTGGGAAAGAAAGCA		
TGF-β1	Forward	GCTGACCCACAGAGAGGAAA	10 μM	60°C
	Reverse	AACTGAACCCGTTGATGTCC		
eNOS	Forward	GATCAGCAACGCTATCACGA	10 μM	60°C
	Reverse	ATACGGCTTGTCACCTCCTG		
18S rRNA	Forward	GTCTGTGATGCCCTTAGATGTC	10 μM	58–60°C
	Reverse	AAGCTTATGACCCGCACTTAC		
Ribosomal protein S29	Forward	CAGGGTTCTCGCTCTTGC	10 μM	60°C
	Reverse	ACTGGCGGCACATATTGAG		
beta-actin	Forward	TGTTACCAACTGGGACGACA	10 μM	60°C
	Reverse	GGGGTGTTGAAGGTCTCAAA		

Forward and reverse primer sequences (5′–3′) used for qPCR to amplify *collagen Iα1*, *collagen Iα2*, *collagen III*, *elastase 2*, *tropoelastin*, *transforming growth factor beta 1 (TGFβ1)*, *endothelial nitric oxide synthase (eNOS)*, *18S rRNA*, *ribosomal protein S29* and *beta-actin*.

2, *tropoelastin*, *endothelial nitric oxide synthase* (*eNOS*) and *transforming growth factor beta 1* (*TGFβ*1). RNA samples were treated with DNase (Qiagen) and were reverse-transcribed into cDNA (Promega); qPCR was performed using the Realplex Real-Time Multiplexing System (Eppendorf, Germany) or the Stratagene MX3000P qPCR machine (Agilent Technologies, USA). Primer sequences (Table 1) were designed using the nucleotide sequences of the genes of interest obtained from the Genbank database (www.ncbi.nlm.nih.gov). Primer and cDNA concentrations and annealing temperatures were optimised (Table 1), and dissociation curves were conducted for each gene to ensure optimal amplification. Samples were analysed in triplicate, with a negative control that did not have template DNA included in each PCR analysis. mRNA levels were normalised against the housekeeping genes *18S rRNA*, *ribosomal protein S29* or *beta-actin* and analysed using the delta C_T (cycle threshold) method, which did not change with alcohol treatment. For each gene, data are expressed relative to the mean mRNA levels obtained for samples of the control animals.

Data analysis

Responses of isolated arterial segments to vaso-constrictors and -dilators were analysed as described previously (Bubb *et al.* 2007; Mazzuca *et al.* 2010). Sigmoidal curves were fitted to the concentration–response data using the least squares methods (GraphPad Prism, GraphPad Software, San Diego, CA, USA) (Bubb *et al.* 2007; Mazzuca *et al.* 2010). From these curves, the concentration of drug that evoked a half-maximal response (EC_{50}), pD_2 ($-\log EC_{50}$) and maximal response (E_{max}) were determined. For endothelium-dependent relaxation, the area-under-the-curve (AUC) was also calculated (Herrera *et al.* 2010) and analysed using 2-way ANOVA (maternal treatment and vasodilator), followed by Tukey's *post hoc* testing. Stress–strain values were derived as described previously (Bubb *et al.* 2007; Mazzuca *et al.* 2010). For normalisation of internal and outside arterial diameters, values were expressed in relation to the diameter at 10 mmHg. An exponential function was fitted to the data and tangential elastic modulus (E_{tang}) was determined from circumferential stress = circumferential stress at 10 mmHg$^{k.strain}$ (k is the slope of the curve). Thus, E_{tang} is proportional to k (Izzard *et al.* 2006). N is the number of animals and statistical significance was accepted as $P < 0.05$. Data are expressed as mean ± SEM and were analysed using one-and two-way ANOVA and unpaired Student's t test (GraphPad Prism).

Results

General effects of maternal ethanol infusion

At the end of the hour of maternal ethanol infusion, ethanol concentration in the maternal and fetal plasma was 117 ± 5 mg dl^{-1} and 108 ± 6 mg dl^{-1}, respectively;

these peak ethanol concentrations in the ewes and fetuses were not significantly different. Ethanol concentrations declined in both ewes and fetuses over 8 h to apparent baseline. Maternal infusion of ethanol had no effect on fetal arterial pressure ($P = 0.93$) or heart rate ($P = 0.53$).

Alcohol exposure had no significant effect on mean arterial pressure (control 38 ± 2 mmHg, alcohol 40 ± 2 mmHg), fetal body weight, on weight of the fetal heart ($P = 0.15$), brain ($P = 0.34$), or kidney ($P = 0.66$), determined at necropsy (data previously reported) (Kenna *et al.* 2011).

Vascular smooth muscle reactivity

Contractile response. We first determined the effects of alcohol on the ability of the fetal arteries to contract upon exposure to high-K^+ PSS. Absolute contraction (tension mN mm^{-1}) to high-K^+ PSS was not different between saline and alcohol groups for fetal coronary, mesenteric, femoral, renal and cerebral arteries.

In coronary artery, U46619 evoked concentration-dependent constriction and the maximum constriction was significantly larger in arteries obtained from alcohol-exposed fetuses (Fig. 1). In contrast, alcohol exposure resulted in a decrease in the responsiveness of cerebral artery to 5-HT (maximal constriction, $P = 0.001$) and renal artery to PE (pD_2 shifted from 6.16 ± 0.07 to 5.56 ± 0.08, $n = 6$, $P = 0.001$). There was no effect of alcohol exposure on the concentration-constriction curves to PE or 5-HT in mesenteric or femoral vessels (Fig. 1).

Relaxation. Endothelium-independent relaxation was tested using SNP. In pre-constricted fetal arteries SNP induced maximal relaxation in all but the coronary artery, in which 10–20% tension remained. Relaxation in segments of coronary, mesenteric, femoral, and cerebral arteries was not affected following alcohol exposure (Fig. 2). However, the sensitivity of the renal artery to SNP was significantly reduced 10-fold following alcohol exposure (pD_2 6.80 ± 0.11 *vs.* 7.90 ± 0.09 in control, $n = 6$, $P < 0.0003$; Fig. 2).

Endothelium-dependent vasodilation

Endothelial stimulation. In the coronary artery, alcohol exposure blunted total endothelium-dependent relaxation induced by BK, with a significant 3-fold shift to the right in the concentration–relaxation curve (Fig. 3, Table 2). Following blockade of NO production, the capacity for maximal dilation was markedly reduced, and inclusion of both L-NAME and indomethacin in the bathing solution revealed that the response attributed to EDHF was significantly attenuated in alcohol-exposed coronary artery, with a significant reduction in maximal relaxation (Fig. 3 and Table 2). Thus, the area-under-curve (AUC) was significantly reduced for NO ($P = 0.02$) and EDHF ($P = 0.03$) in coronary artery (Fig. 4*A*).

In contrast, in mesenteric segments, exposure to alcohol resulted in a marked increase in overall endothelium-dependent vasodilation, with a 10-fold left shift in the curve and a significant increase in pD_2, and this persisted in the presence of L-NAME and in L-NAME

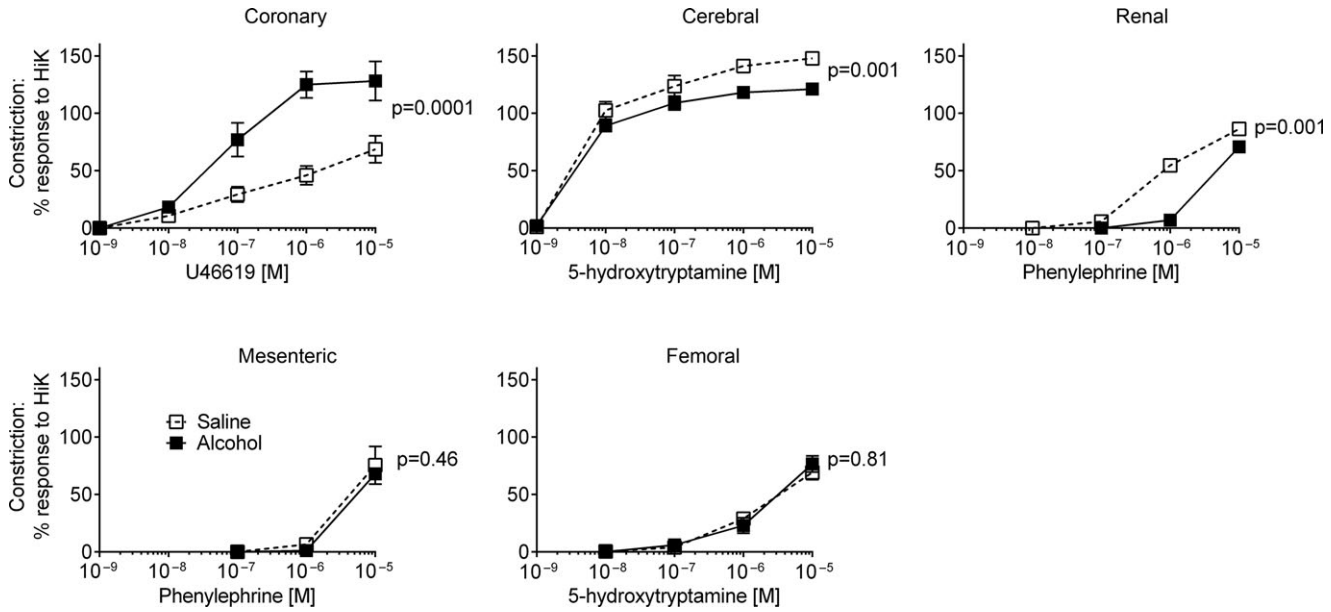

Figure 1. Effects of alcohol exposure in late pregnancy on fetal vascular smooth muscle contraction
Constriction (relative to high-K^+ PSS) was enhanced in coronary artery, suppressed in cerebral and renal segments, while mesenteric and femoral arteries were unaffected. Fetuses from 6 control ewes (saline) and 6 alcohol-treated ewes. Values of *P* refer to E_{max}.

plus indomethacin (Fig. 3 and Table 2). Overall vasodilator capacity was also increased by alcohol in the renal artery, but this reflected a 7-fold enhancement of NO bioavailability alone, as there was no difference between treatments in the presence of blockers of NO and prostanoid (Fig. 3). AUC showed up-regulation of NO-mediated relaxation for mesenteric ($P = 0.04$) and renal ($P = 0.04$) arteries and also for EDHF in mesenteric ($P = 0.03$) artery (Fig. 4*A*).

Endothelium-dependent relaxations in cerebral and femoral arteries induced by BK and ACh, respectively, were unaffected by alcohol exposure (Fig. 4*A*, Table 2).

Tone development. When L-NAME was added to the superfusing solution, control coronary, cerebral and femoral segments developed substantial tone (~40% of a high potassium contraction). This tone was similar in cerebral and femoral arteries from saline- and alcohol-exposed fetuses. However, L-NAME-induced tone did not develop in coronary artery from alcohol-exposed fetuses (Fig. 4*B*).

Passive mechanical wall properties

Increasing the pressure in segments of fetal coronary, mesenteric, femoral, renal and cerebral arteries induced a progressive increase in vessel diameter. For all arteries tested, the increase was significantly greater in control vessels *versus* arteries from alcohol-exposed fetuses (Fig. 5*A*). Stress–strain curves for arteries from alcohol-exposed fetuses were shifted to the left, and k was significantly increased, an indication of increased stiffness, in all arterial segments following exposure to alcohol (Fig. 5*B* and Table 3). Wall thickness and lumen diameter were determined at 50 mmHg, and there was no effect of alcohol on these dimensions or on lumen diameter:wall thickness ratio in any of the arteries studied (data not shown).

Short-term alcohol treatment

Maternal infusion of alcohol for 3 days between 131 and 133 days of gestation had no effect on smooth muscle contraction, endothelium-independent relaxation,

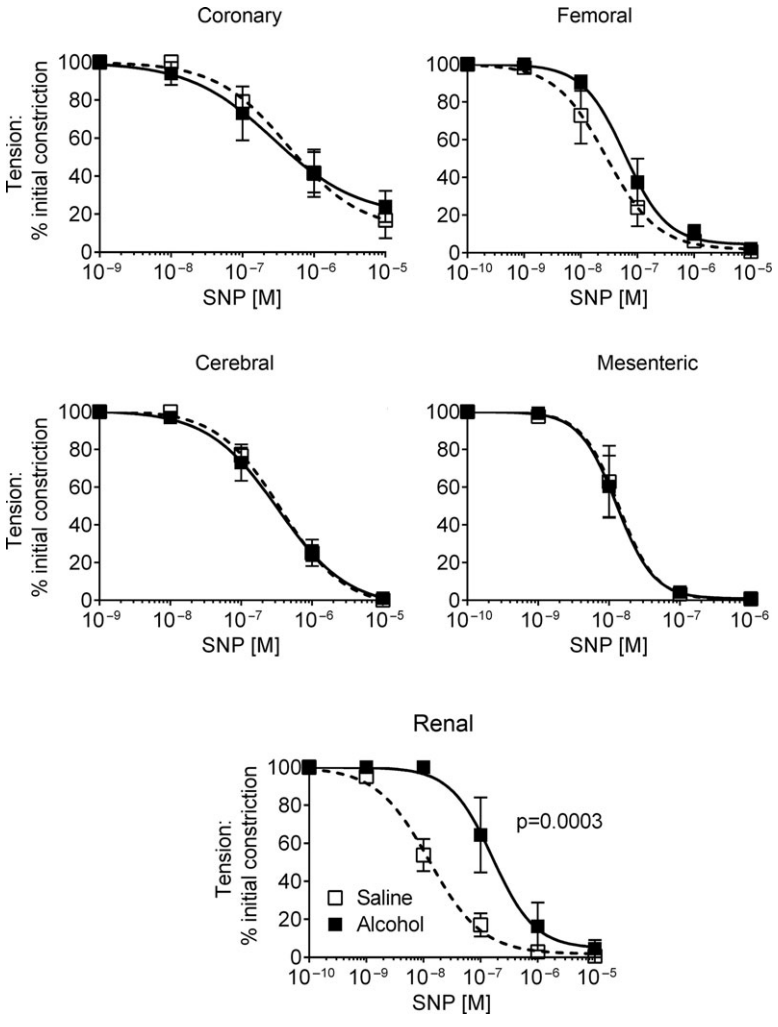

Figure 2. Endothelium-independent relaxation in fetal arteries
Alcohol exposure in late pregnancy significantly reduced the pD_2 to nitroprusside in fetal renal artery but was without effect in the other arteries. $N = 6$ in each group. The P value refers to pD_2.

endothelium-dependent relaxation or stiffness in fetal coronary artery (data not shown).

Quantitative real-time PCR analysis for selected gene expression

Relative *collagen Ia1* mRNA level was significantly increased in the cerebral artery ($P = 0.02$) of alcohol-exposed fetuses (Fig. 6), while *collagen Ia2* and *collagen III* mRNA levels in the five selected fetal arteries were unaffected. Relative *tropoelastin* mRNA level was increased only in cerebral artery ($P = 0.0003$), and *eNOS* mRNA level was decreased in coronary artery ($P = 0.002$) of alcohol-exposed fetuses compared with controls (Fig. 6). Relative *TGF-β1* and *elastase 2* mRNA levels in the fetal arteries were not altered by alcohol exposure (Fig. 6).

Discussion

This study demonstrates significant widespread changes in vasodilator function and arterial stiffness in the fetus following daily alcohol exposure during late pregnancy. Effects on vasodilator function were region dependent. Endothelium-dependent vasodilation was markedly reduced in coronary artery, accompanied by a decrease in *eNOS* mRNA. In contrast, vasodilation was enhanced in mesenteric and renal resistance arteries. There was a striking and widespread increase in stiffness in all arteries studied, which was accompanied by an increase in elastic modulus, suggesting changes in vessel wall composition. These changes were the result of relatively long-term (30 days) exposure to alcohol, and were not produced by short (3 day) exposure in late pregnancy.

Endothelium-dependent vasodilator function is critical for the maintenance of tissue perfusion and for matching blood flow with organ activity. Endothelial dysfunction precedes the development of hypertension, atherosclerosis, stroke and is prominent in inflammatory diseases such as diabetes (Vanhoutte *et al.* 2009). Endothelial vasodilator function is affected in many forms of intrauterine insult (Poston, 2007), to which prenatal

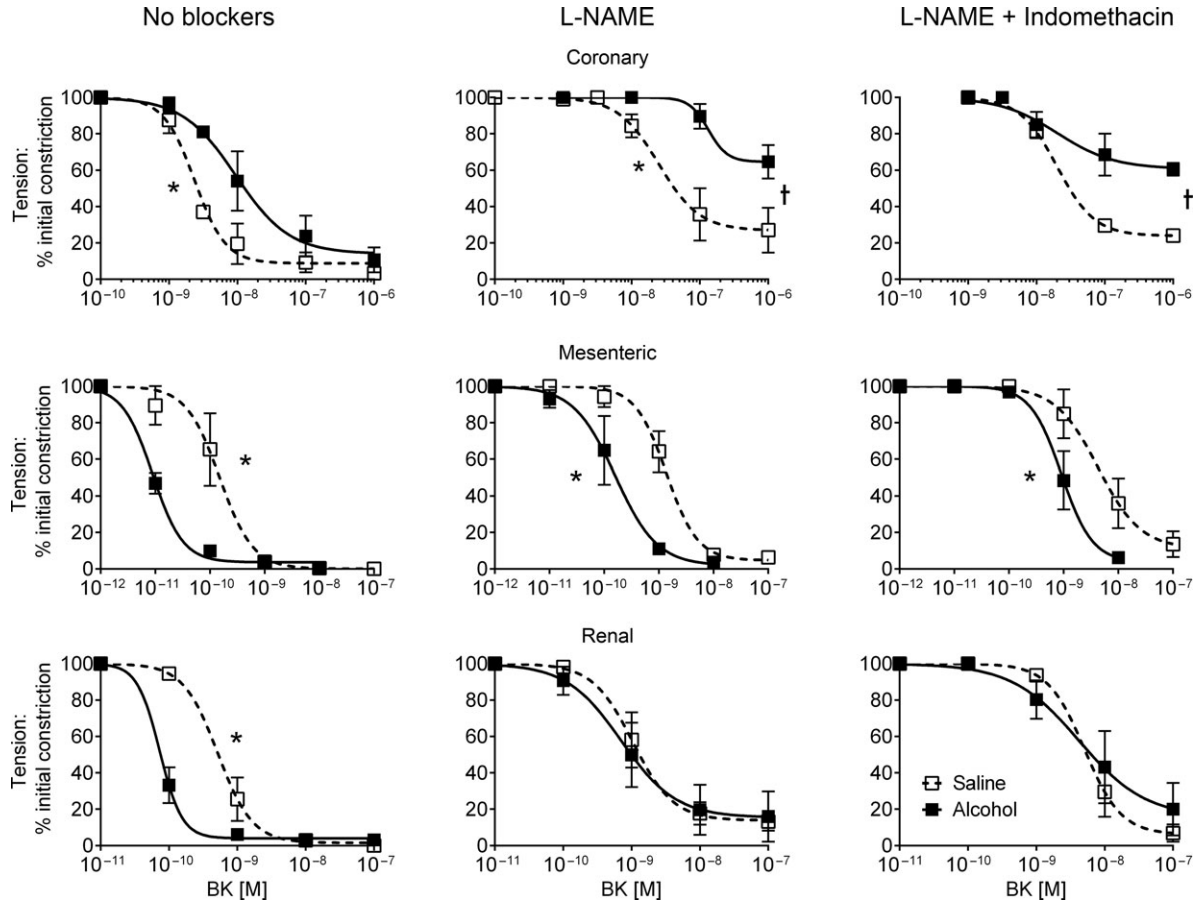

Figure 3. Endothelium-dependent relaxation in fetal arteries
Endothelium-dependent relaxation was significantly reduced in fetal coronary artery, while relaxation was enhanced in mesenteric and renal arteries exposed to alcohol. $N = 6$ in each group. *Significant difference in pD_2, †significant difference in E_{max}.

Table 2. Values of pD_2 (−log EC_{50}) and E_{max} (% maximal constriction) responses to endothelial stimulation in small coronary, cerebral, renal, mesenteric and femoral arteries of fetal sheep following alcohol exposure

	pD_2			E_{max} (% maximal constriction)		
	Saline	Alcohol	*P*	Saline	Alcohol	*P*
No blocker						
Coronary	8.63 ± 0.08	8.02 ± 0.18*	0.01	9 ± 4	14 ± 9	0.62
Mesenteric	9.82 ± 0.17	11.05 ± 0.04*	0.0001	0 ± 0	4 ± 2	0.21
Renal	9.29 ± 0.10	10.14 ± 0.22*	0.006	1 ± 3	4 ± 3	0.50
Femoral	7.45 ± 0.09	7.53 ± 0.12	0.61	1 ± 3	4 ± 4	0.56
Cerebral	9.66 ± 0.18	9.48 ± 0.08	0.38	8 ± 7	7 ± 4	0.90
+ L-NAME						
Coronary	7.60 ± 0.23	6.88 ± 0.09*	0.02	27 ± 9	65 ± 6*	0.005
Mesenteric	8.86 ± 0.12	9.80 ± 0.16*	0.0008	5 ± 6	2 ± 6	0.73
Renal	8.98 ± 0.12	9.15 ± 0.25	0.55	14 ± 8	15 ± 10	0.96
Femoral	6.75 ± 0.10	6.67 ± 0.11	0.56	3 ± 4	11 ± 5	0.24
Cerebral	8.79 ± 0.31	8.73 ± 0.45	0.94	57 ± 11	63 ± 10	0.70
+ L-NAME + Indo						
Coronary	7.70 ± 0.03	7.70 ± 0.40	0.99	24± 10	61 ± 8*	0.02
Mesenteric	8.37 ± 0.13	9.04 ± 0.11*	0.003	11 ± 10	4 ± 8	0.60
Renal	8.30 ± 0.14	8.38 ± 0.38	0.85	6 ± 8	16 ± 6	0.34
Femoral	5.97 ± 0.40	6.00 ± 0.41	0.96	19 ± 18	32 ± 20	0.64
Cerebral	8.43 ± 0.56	6.61 ± 0.67	0.06	71 ± 10	39 ± 21	0.20

Daily maternal infusion for 1 h of saline ($n = 6$) or alcohol ($n = 6$). Concentration–relaxation curves were first obtained in control solution and then in the presence of L-NAME to block NO production, and in L-NAME + indomethacin (Indo) to additionally block prostanoid production. The statistical significance of the data is indicated by *P*; *significant difference between saline and alcohol treatment.

alcohol exposure can now be added. Several effects of alcohol consumption on endothelial function in the adult cardiovascular system have been reported. Thus, while alcohol directly constricts canine aorta denuded of endothelium (Yang *et al.* 2001), it normally produces relaxation of rat aorta by inducing endothelial NO production (Turcotte *et al.* 2002). In rat mesenteric artery, alcohol increased endothelin receptor A (ET_A) expression and enhanced constriction, while in the kidney, alcohol decreased endothelial ET_B expression with a consequent increase in NO production and renal vessel dilation (Tirapelli *et al.* 2006). Alcohol can also increase endothelin production by human endothelial cells (Yeligar *et al.* 2009). Thus, the effect of alcohol depends on the vascular bed. It must also be borne in mind that, while the initial endothelial response to a variety of stresses such as oxidative stress or inflammation is a compensatory increase of NO production, this does not always persist with chronic stress. These issues may explain the differences in endothelial responses observed in different arteries of the

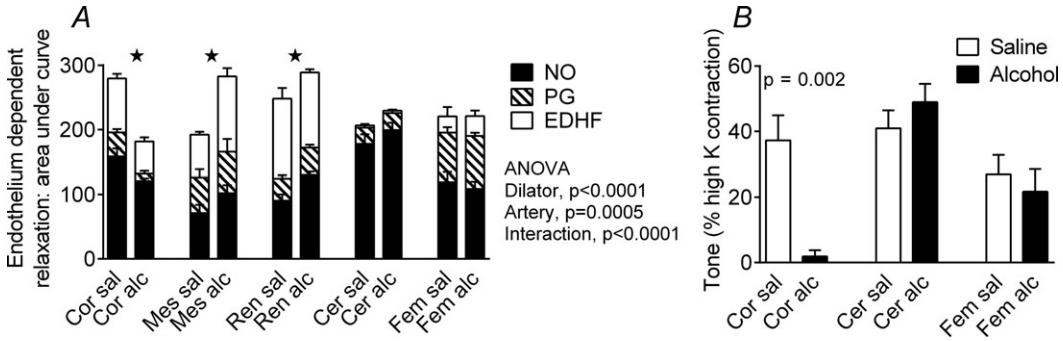

Figure 4. Integrated endothelium-dependent relaxation and tone development in fetal arteries
A, area under the endothelium-dependent relaxation curves for NO, PG and EDHF in the five fetal arteries studied. Alcohol exposure during late pregnancy changed NO and/or EDHF contraction in coronary, mesenteric and renal, but not in cerebral or femoral arteries. *B*, basal tone development following inclusion of L-NAME in the superfusing solution. $N = 6$ per group. *Significant difference in overall AUC between saline and alcohol exposure.

alcohol-exposed fetus, as in the present study. Whereas NO and EDHF functions were markedly reduced in coronary artery, the function of these dilators was enhanced in mesenteric and renal arteries. The endothelium is well known to modulate sensitivity to vasoconstrictors. It may also be that the increase in sensitivity of coronary artery to the contractile effect of U46619 and the marked reduction in the constrictor effect of phenylephrine in the renal artery are related to fundamental functional changes in the endothelium as a result of alcohol exposure.

The small vessels derived from the fetal cerebral and femoral arteries were remarkably resistant to alcohol exposure, in terms of endothelium-dependent or -independent changes in vascular tone. Cerebral blood vessels have been the focus of most studies targeting the effects of intrauterine alcohol exposure on vascular function to date and the findings have been variable. An elegant study by Parnell and colleagues in a sheep model similar to the one used in our study found that plasma alcohol concentration of ∼185 mg dl^{-1} increased global cerebral blood flow, but there was significant variability between different regions of the brain (Parnell *et al.* 2007). In another sheep study in which alcohol was infused into ewes in early pregnancy and the lambs were studied postnatally, alcohol had no effect on cerebral blood flow but the response to hypoxia was blunted (Gleason *et al.* 1997). Clearly, the effects of alcohol on autoregulatory processes in the brain require further study.

The kidney has a central role in cardiovascular and blood pressure control. Feeding pregnant rat dams with alcohol-containing food resulted in a significant increase in blood pressure in the offspring (Turcotte *et al.* 2002; Gray *et al.* 2010), although there was no effect on this variable in our fetal sheep (Gray *et al.* 2008), or in children of mothers who consumed alcohol during pregnancy (Morley *et al.* 2010). Nephron endowment was reduced in postnatal rats (Gray *et al.* 2010) and in our fetal sheep (Gray *et al.* 2008) following intrauterine alcohol exposure. In the present study smooth muscle responsiveness to SNP in the renal artery was reduced by an order of magnitude following alcohol exposure. This was an unexpected finding, as this variable is usually preserved following intrauterine compromise of fetal development (Payne *et al.* 2003; Bubb *et al.* 2007; Mazzuca *et al.* 2010). However, impaired responsiveness to SNP in cerebral artery of offspring exposed to maternal low protein diet is underpinned by reduced levels of guanylate cyclase and cGMP (Lamireau *et al.* 2002). Remarkably, in our study, despite impaired smooth muscle responsiveness to NO in fetal renal artery, total endothelial vasodilator function was significantly enhanced by alcohol exposure, which can be attributed to an increased contribution of NO. The enhanced endothelium-derived NO response was probably underestimated in our study, as the functional response must overcome the reduced sensitivity of the muscle to this vasodilator. Alternatively, up-regulation of the L-NAME-sensitive component may be underpinned

Table 3. Elastic modulus, *k*, for small coronary, cerebral, renal, mesenteric and femoral arteries of fetal sheep following daily maternal infusion for 1 h of saline ($n = 6$) or alcohol ($n = 6$)

	Saline	Alcohol	*P*
Coronary	16.4 ± 1.7	31.4 ± 3.8*	0.005
Cerebral	19.7 ± 2.3	33.5 ± 4.8*	0.002
Renal	15.7 ± 1.2	26.6 ± 1.9*	0.0007
Mesenteric	21.3 ± 1.9	33.7 ± 2.3*	0.002
Femoral	35.4 ± 2.5	63.3 ± 7.1*	0.004

The statistical significance of the data is indicated by *P*; *significant difference between saline and alcohol treatment.

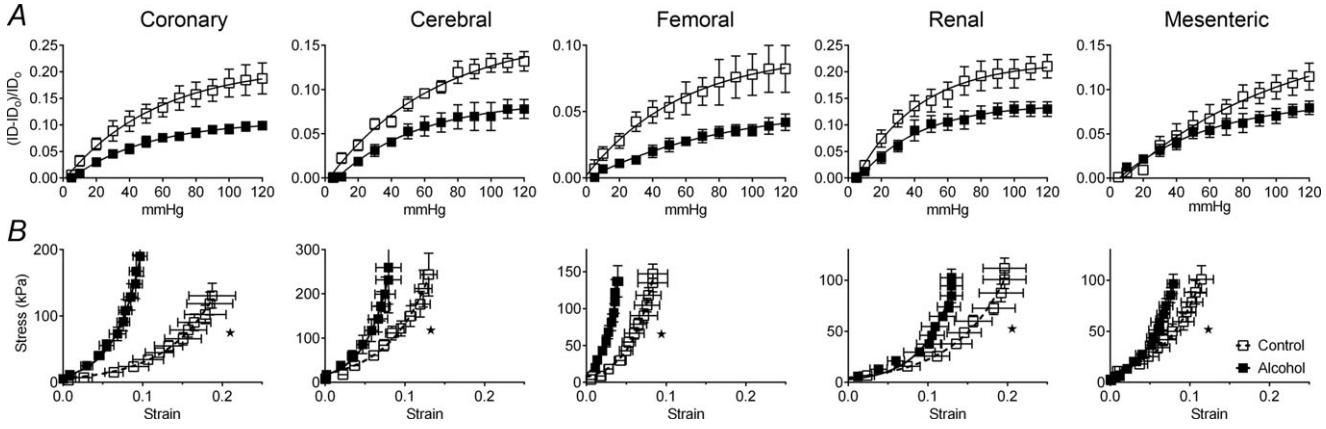

Figure 5. Effect of alcohol on arterial stiffness in the fetus
A, increasing pressure in segments of fetal artery induced an increase in lumen diameter. This response was blunted in arteries from alcohol-exposed fetuses. Alcohol *versus* control was significantly different ($P < 0.0001$ for all arteries). *B*, segments from all 5 vascular beds were significantly stiffer following alcohol exposure. $N = 6$ per group. *Significant difference in *k* between control and alcohol-exposed fetuses.

by an additional endothelial vasodilator, namely, nitroxyl (HNO). HNO is a one-electron reduced and protonated form of NO, can target signalling pathways distinct from NO, and is resistant to scavenging by superoxides (Andrews *et al.* 2009; Bullen *et al.* 2011).

Arterial stiffness was significantly increased in fetal resistance arteries of all five beds studied. The increase was most marked in coronary, renal and cerebral vessels. This is the first study to show that alcohol exposure potently increases arterial stiffness in the fetus. In adult males, progressive alcohol consumption is associated with an increase in pulse-wave velocity (PWV) (Kurihara *et al.* 2004) and augmentation index (van Trijp *et al.* 2005), reliable indicators of decreased arterial compliance (DeLoach & Townsend, 2008). Alcohol consumption by women at any stage of pregnancy was associated with a persistent increase in PWV in their children at 9 years of age (Morley *et al.* 2010). In the present study, *collagen Ia1* mRNA expression was increased following alcohol exposure in fetal cerebral artery. Elastin is laid down extensively during the perinatal period of development (Martyn & Greenwald, 1997), making it especially vulnerable to intrauterine insult. Elastin has a very long half-life, up to 40 years in humans, indicating that perturbations in this extracellular matrix protein in early life are likely to have long lasting effects (Martyn & Greenwald, 2001). *Tropoelastin* mRNA was increased, but only in alcohol-exposed cerebral artery. The distensibility and stiffness of the arterial wall depends not only on the amount of collagen and elastin, but on the relative thickness of the fibres and their organisation (Zieman *et al.* 2005). While direct determination of collagen and elastin protein content would be of interest, the small size of these vessels precluded direct measurement here. None-the-less, our observations are important because a reduction in arterial compliance is associated with an increased risk of cardiovascular disease (Laurent *et al.* 2001).

Structural and functional maturation of many organs is high in late pregnancy. DNA replication and cell proliferation are vulnerable to disruptions in methionine metabolism and protein and DNA methylation. Men consuming 24 g of alcohol for 2 weeks had a reduction in folate and vitamin B12 and an increase in homocysteine (Gibson *et al.* 2008). Homocysteine is critically important

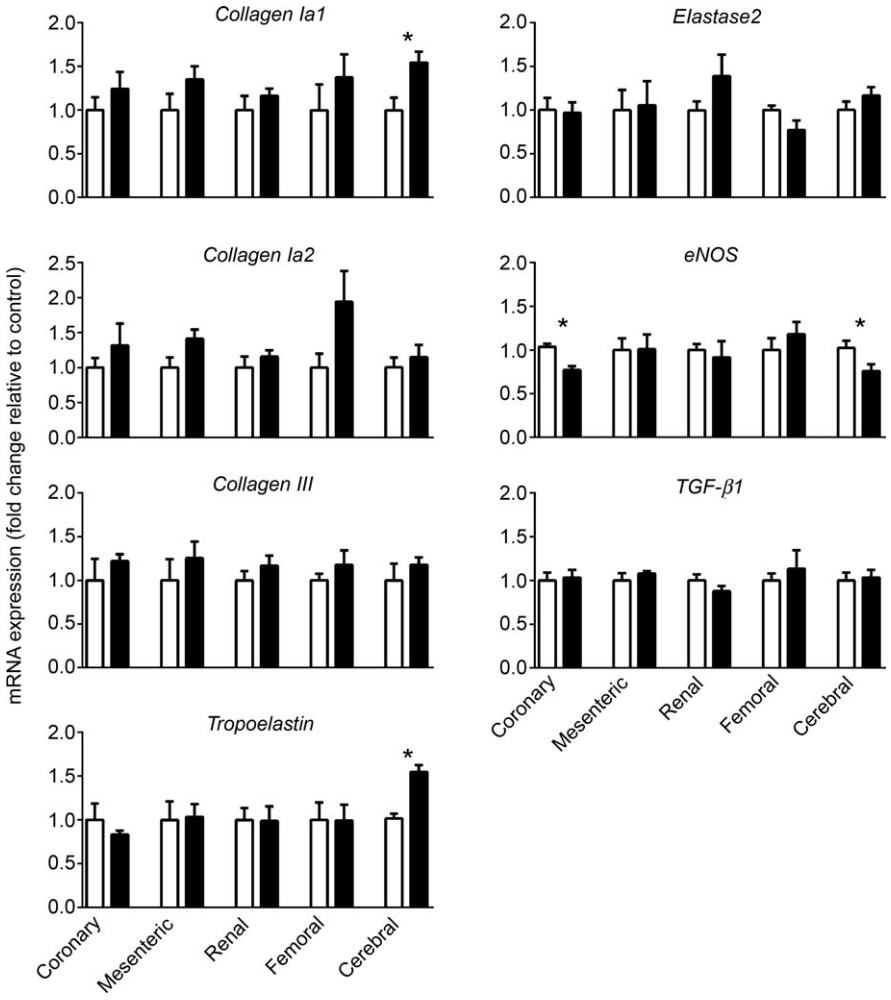

Figure 6. mRNA gene expression in arteries of fetuses exposed to alcohol during pregnancy
Prenatal alcohol exposure increased *collagen Ia1* mRNA level in fetal cerebral artery, but there was no change in *collagen Ia2* or *collagen III*. Prenatal alcohol exposure increased *tropoelastin* mRNA expression in cerebral artery, while decreasing *eNOS* mRNA expression in coronary arteries. There was no difference in *TGFβ1* or *elastase 2* mRNA level between alcohol and control groups. Open bars, saline ($n = 4$–5); filled bars, alcohol ($n = 4$–5). *Significant difference between saline and alcohol exposure.

in methionine metabolism and protein and DNA methylation (Lutz, 2008). Epigenetic modifications predispose to life-long cardiovascular disease and maternal hyperhomocysteinemia is associated with an increased risk of congenital heart disease in the offspring (van Driel *et al.* 2008). Homocysteine can also inhibit vascular endothelial cell growth (Chang *et al.* 2008), and is involved in the methylation of arginine, giving rise to asymmetric dimethylargine (ADMA) production, an endogenous inhibitor of NOS. In addition, alcohol can increase oxidative stress (Altura & Gebrewold, 2002), which also reduces NO bioavailability and endothelium-dependent vasodilator function. On the other hand, modest alcohol consumption can reduce cardiovascular risk as a result of enhanced NOS synthesis (Di Castelnuovo *et al.* 2006). This varied palette of possibilities forms the basis for the wide range of effects observed in the present study.

We conclude that daily maternal alcohol administration during the last trimester equivalent induces widespread vascular adaptations in the fetus, with increased arterial stiffness, present in all five vascular beds under study. In contrast, endothelium-dependent vasodilator responses to fetal alcohol exposure are not uniform across different vascular beds, with suppressed (coronary) or enhanced (renal and mesenteric) dilatation shown here for the first time. Maladaptive changes in the fetal vasculature in response to alcohol exposure may persist postnatally, thereby increasing the risk of cardiovascular disease in adulthood. Of alarming concern is that these marked changes in vascular function occur at levels of exposure that are not associated with overt signs of FAS.

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Additional information

Competing interests

None declared.

Author contributions

Study conception and design: H.C.P., M.T., H.A.C., D.W.W., R.H. Data collection, analysis and interpretation: H.C.P., M.T., K.K., F.S., H.A.C. Manuscript preparation and intellectual input: H.C.P., M.T., J.F.B., R.H., D.W.W., H.A.C., K.K., F.S., A.B. All authors have approved the final version.

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