

# Effects of Maternal Sildenafil Treatment on Vascular Function in Growth-Restricted Fetal Sheep

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**Objective**—The objective of this study was to investigate the effect of intravenous maternal sildenafil citrate (SC) administration on vascular function in growth-restricted fetal sheep.

**Approach and Results**—Fetal growth restriction (FGR) results in cardiovascular adaptations that redistribute cardiac output to optimize suboptimal intrauterine conditions. These adaptations result in structural and functional cardiovascular changes, which may underlie postnatal neurological and cardiovascular sequelae. Evidence suggests SC, a potent vasodilator, may improve FGR. In contrast, recent clinical evidence suggests potential for adverse fetal consequence. Currently, there is limited data on SC effects in the developing fetus. We hypothesized that SC in utero would improve vascular development and function in an ovine model of FGR. Preterm lambs (0.6 gestation) underwent sterile surgery for single umbilical artery ligation or sham (control, appropriately grown) surgery to replicate FGR. Ewes received continuous intravenous SC (36 mg/24 h) or saline from surgery until 0.83 gestation. Fetuses were delivered and immediately euthanized for collection of femoral and middle cerebral artery vessels. Vessel function was assessed via *in vitro* wire myography. SC exacerbated growth restriction in growth-restricted fetuses and resulted in endothelial dysfunction in the cerebral and femoral vasculature, irrespective of growth status. Dysfunction in the cerebral circulation is endothelial, whereas smooth muscle in the periphery is the origin of the deficit.

**Conclusions**—SC crosses the placenta and alters key fetal vascular development. Extensive studies are required to investigate the effects of SC on fetal development to address safety before additional use of SC as a treatment.

**Visual Overview**—An online [visual overview](#) is available for this article. (*Arterioscler Thromb Vasc Biol.* 2019;39:731-740. DOI: 10.1161/ATVBAHA.119.312366.)

**Key Words:** fetus ■ myography ■ placenta ■ sheep ■ sildenafil citrate

Fetal growth restriction (FGR) is the failure of a fetus to reach their genetic growth potential.<sup>1</sup> FGR is the second highest cause of perinatal death, complicating ≈5% to 10% of all pregnancies<sup>2</sup> and increasing stillbirth risk 20-fold.<sup>3</sup> After birth, FGR infants have an increased risk of both short- and long-term disease.<sup>4</sup> FGR most commonly occurs because of placental insufficiency, causing impaired placental blood flow and fetal hypoxia. In response to hypoxia, the fetus mounts a compensatory cardiovascular response to redistribute blood supply to important organs (eg, brain) at the expense of the periphery.<sup>5</sup> Despite the redistribution of blood flow, FGR infants are at greater risk of developing neurological and cardiovascular sequelae compared with their appropriately grown (AG) counterparts.<sup>6</sup>

Brain-sparing is achieved via vasoconstriction of nonessential vascular beds, such as the periphery, this mechanism is initiated by chemoreflexes and sustained by reduced NO bioavailability.<sup>7</sup> Long-term chronic fetal hypoxia and persistent alteration in vascular resistance also cause vascular developmental deficits, such as increased thickening and stiffness

of the aorta and carotid vessels and endothelial dysfunction,<sup>8</sup> an early marker of cardiovascular disease.<sup>9</sup> NO is produced by eNOS (endothelial NO synthase) located within the endothelial layers of blood vessels and is integral to local blood pressure control, acting as an endogenous vasodilator to mediate vascular diameter and blood flow.<sup>10</sup> It is well described that the FGR fetus has impaired NO-mediated vasodilation which can be attributed to NOS deficiency and decreased NO bioavailability.<sup>9</sup>

Despite significant perinatal complications associated with FGR, there is currently no routine therapy to improve outcomes. Sildenafil citrate (SC) is a PDE (phosphodiesterase) 5 inhibitor which causes smooth muscle relaxation in blood vessels to increase vasodilation and subsequently, blood flow. SC has been proposed as a therapy in FGR pregnancies by acting to vasodilate placental blood vessels, which are abundant in PDE5, and increase placental blood flow.<sup>11</sup> In turn, improved placental blood flow is hypothesized to increase oxygen and nutrient transfer to the fetus, increasing fetal weight. Promising results from preclinical studies<sup>12,13</sup>

Received on: August 16, 2018; final version accepted on: February 15, 2019.

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The online-only Data Supplement is available with this article at <https://www.ahajournals.org/doi/suppl/10.1161/ATVBAHA.119.312366>.

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*Arterioscler Thromb Vasc Biol* is available at <https://www.ahajournals.org/journal/atvb>

DOI: 10.1161/ATVBAHA.119.312366

**Nonstandard Abbreviations and Acronyms**

<b>AG</b>	appropriately grown
<b>AG<sub>sc</sub></b>	SC-treated appropriately grown
<b>eNOS</b>	endothelial nitric oxide synthase
<b>FEM</b>	femoral artery
<b>FGR</b>	fetal growth restriction
<b>FGR<sub>sc</sub></b>	SC-treated fetal growth restricted
<b>MCA</b>	middle cerebral artery
<b>PDE5</b>	Phosphodiesterase 5
<b>SC</b>	sildenafil citrate
<b>sGC</b>	soluble guanylate cyclase
<b>SNP</b>	sodium nitroprusside
<b>STRIDER</b>	Sildenafil Therapy in Dismal Prognosis Early-Onset Intrauterine Growth Restriction
<b>SUAL</b>	single umbilical artery ligation

have underpinned a set of worldwide multicenter clinical trials involving the United Kingdom/Ireland, the Netherlands, Australia/New Zealand, and Canada. The Sildenafil therapy in dismal prognosis early-onset intrauterine growth restriction (STRIDER) consortium aimed to determine if maternal SC treatment increases gestation length, placental function, and fetal growth.<sup>14</sup> Although results from some countries have now been reported and show no benefit of SC (AusNZ), the Dutch STRIDER was recently terminated before completion because of increased neonatal mortality in SC-treated infants compared to control.<sup>15</sup>

Maternally-administered SC crosses the placenta into the fetal circulation,<sup>16</sup> where SC has potential to mediate direct cardiovascular effects. We and others have demonstrated dysfunction of the NO pathway in FGR when compared to AG.<sup>17,18</sup> Therefore, given the vascular adaptations associated with FGR, predisposition toward endothelial dysfunction and the NO-mediated mechanism of SC action, we think investigation into potential fetal effects of SC exposure is a critical unknown. Herein, we utilized our established ovine model of FGR, which involves single umbilical artery ligation (SUAL) to determine fetal effects of maternal sildenafil administration. SUAL results in placental atrophy, subsequent reduction of placental function and the induction of clinical FGR features (decreased weight, brain-sparing).<sup>19</sup> We aimed to investigate the effect of maternal SC treatment on fetal vascular structure and function in growth-restricted fetuses. We hypothesized that maternal SC crosses the placenta into the fetal circulation, improves fetal growth, and results in functional improvement of NO-mediated vasodilation within the peripheral vasculature.

## Materials and Methods

Experiments were approved by the Monash Medical Centre animal ethics committee (MMCA2016/01) in accordance with guidelines established by the National Health and Medical Research Council of Australia.

## Animals

Twin bearing Border-Leicester pregnant ewes (n=13) underwent surgery on day 88 to 90 days gestation (term approximately 148 days

gestation) to induce severe, early-onset FGR via SUAL, as previously described.<sup>19</sup> Previous studies by our group have investigated differences between early (89 days) and late (105 days) SUAL and have found that early SUAL resulted in increased FGR and brain-sparing.<sup>19</sup> As criteria for the STRIDER was diagnosis of early and severe FGR,<sup>20</sup> we chose to use the early SUAL model in the current study. Briefly, anesthetized ewes underwent surgery in which 1 fetus (randomly selected) underwent SUAL to induce FGR. The umbilical cord of the twin fetus was manipulated but not ligated (control, AG). The fetuses were returned to the uterus, and catheters inserted into a maternal jugular vein for antibiotic and treatment administration and a carotid artery for blood sampling.

## Experimental Procedures

Antibiotics were administered to the ewe (Ampicillin, 1g and Engemycin 5mL IV) for 3 days after surgery via the maternal jugular vein catheter.

## Maternal Sildenafil/Saline Administration

On day 3 postsurgery, after completion of the antibiotics regimen, ewes were randomized to receive either SC (n=7, 36 mg/d) or saline (n=6) infusion via a pump (CADD-Legacy 1 Pump; Smiths Medical, Australia) connected to the maternal jugular vein catheter and fastened to the back of the ewe under netting. Twin fetuses within the ewes were subsequently randomized into groups; FGR and AG or SC-treated FGR (FGR<sub>sc</sub>) and SC-treated AG (AG<sub>sc</sub>). SC and saline administration occurred continuously for ≈5 weeks until postmortem (126 days gestation). This treatment regimen was used to reflect the dose chosen and used in the STRIDER trial.<sup>14</sup>

## Postmortem

At 126 days gestation, fetuses from all groups were euthanized via pentobarbital sodium overdose to the ewe (100 mg/kg IV 153 Valabarb; Jurox, Rutherford, Australia). Third-order middle cerebral arteries (MCA) and femoral arteries (FEM) were carefully and immediately dissected for functional assessment (in vitro wire myography, see below).

## Soluble Guanylate Cyclase Protein Level

sGC (soluble guanylate cyclase) is an enzyme present in smooth muscle cells and converts GTP into active cGMP. cGMP is the key intracellular molecule that is ultimately responsible for smooth muscle relaxation and subsequent vasodilation. We quantified sGC protein levels via Western blot.

Snap-frozen femoral vessel samples were homogenized in 0.1M Tris/HCL lysis buffer with 0.5% Triton X with 10 μL/mL phenylmethylsulphonyl fluoride. Total protein content of the supernatant was determined via the Lowry method using a Bio-Rad DC (detergent compatible) Protein Assay kit (Bio-Rad, CA). Protein samples (30 μg) in 4x sodium dodecyl sulfate (SDS) sample buffer (EMD Millipore, CA) were heat denatured, separated using 10% SDS-PAGE, and transferred to nitrocellulose membrane (Protran BA-85 nitrocellulose membrane, Schleicher and Schuell, Germany). Membranes were then incubated in primary antibody (rabbit anti-sGCb1 antibody, 1:500; Cayman or b-actin 1:1000, Cell Signalling Technologies) with 5% skim milk block overnight at 4°C. The protein was then detected by goat anti-rabbit IgG-HRP, 1:10,000 (Santa Cruz Biotechnology), and visualized with Chemiluminescence reagent LumiGLO (Cell Signalling Technologies, Danvers, MA) using Chemidoc XRS (Biorad, CA). The membrane image was analyzed using ImageJ analysis software.<sup>21</sup> The protein expression level was calculated from the average density of the sGC band divided by the average density of the beta-actin band.

## In Vitro Wire Myography

Wire myography allows for the interrogation of vascular response and reactivity to vasoactive agents.<sup>22</sup> Third-order MCA and FEM

were dissected and placed in chilled Krebs Solution: mmol/L: NaCl 118.845, KCl 4.69, MgSO<sub>4</sub> 1.156, KH<sub>2</sub>PO<sub>4</sub> 1.175, NaHCO<sub>3</sub> 25.2, D-glucose 12, EDTA 0.03, and CaCl<sub>2</sub> 2.5 and bubbled with carbogen (95% O<sub>2</sub> and 5% CO<sub>2</sub>). Two-millimeter dissections of each vessel were threaded with a 40 μm diameter wire, with care to avoid touching the endothelium, and mounted onto a 4-chamber mire myograph (Multi-Wire Myograph System 610M, DMT, Denmark). The force of arterial resistance was normalized (0.9 at 5.3 kPa) to mimic fetal lamb blood pressure (≈50 mm Hg).<sup>23</sup> Initial contractile capacity was assessed in response to potassium (K<sup>+</sup>) physiological saline (0.12 M), and endothelial vasodilation ability was assessed with acetylcholine (Sigma A6625; Sigma-Aldrich). Collection and mounting of 2 mm dissections were repeated until viability of the vessel was determined via response to K<sup>+</sup> solution and acetylcholine.

### Assessment of Atrial Vasodilation Via Myography

Wire-mounted arteries were precontracted with K<sup>+</sup> physiological saline (0.6M). K<sup>+</sup> physiological saline allowed for assessment of smooth muscle contractility and subsequent assessment of vasodilation of both MCA and FEM arteries in response to acetylcholine (Sigma A6625, 10<sup>-9</sup>–10<sup>-4</sup> mol/L; Sigma-Aldrich), sodium nitroprusside (SNP, Sigma 71778, 10<sup>-10</sup>–10<sup>-4</sup> mol/L; Sigma-Aldrich). Assessment of vasodilation in response to SC was also performed on MCA (SC, Sigma PZ0003, 10<sup>-9</sup>–10<sup>-4</sup> mol/L; Sigma-Aldrich). Response to drugs was performed via a similar method. After plateau of a submaximal contractile response from vasodilator, an initial dose was added to the bath to a concentration of 10<sup>-10</sup> mol/L or 10<sup>-9</sup> mol/L depending on the curve. Vessels were then exposed to progressively higher concentration (increased by a factor of 10 with each administration) until a final bath concentration of 10<sup>-4</sup> mol/L was achieved. SNP, acetylcholine, and sildenafil bath concentration was increased after plateau. Vasodilation response curves were performed on the same vessel. Vessels were rested at baseline for 20-minute rest between each response curve.

Average force exerted by the artery during each incubation was recorded. Individual results were averaged for drug concentration to create dose-response dilation curves. Relaxation curves were analyzed using an agonist line of best-fit assessments to determine overall maximal relaxation (%Rmax) and sensitivity (half-maximal effective concentration [pEC<sub>50</sub>]).

The contribution of PDE5 to vasodilation within the MCA was calculated by subtracting the area under the curve in response to SC from the area under the curve in response to acetylcholine.<sup>24</sup>

### Plasma Sildenafil Measurement

Maternal plasma samples were collected on alternate days from day 1 postsurgery until postmortem. In a subgroup of animals exposed to SC treatment, fetal plasma samples were collected at postmortem. Approximately 1 microliter of plasma samples were collected and analyzed for SC concentration via liquid chromatography-mass spectrometry by Thermo Fisher Australia using a protocol established by Vos et al.<sup>25</sup> SC is a PDE 5 inhibitor, which blocks the breakdown of cGMP to GMP in blood vessels. Increased intracellular smooth muscle cGMP mediates effects on downstream pathways to ultimately cause decreased vascular resistance,<sup>26</sup> increased vasodilation, and subsequently, increased blood flow.

### Statistical Analysis

Data are for animal weight, ratios and sildenafil concentration presented as mean±SD. Data for myography analysis are presented as mean±SEM. Maximal contraction to high K<sup>+</sup> was analysed using a 1-way ANOVA, and a Tukey post hoc test was used to compare between groups (Prism 7 for Mac OS X, GraphPad Software). Body weight and brain to body weight ratio were analysed using a 2-way ANOVA and Tukey post hoc test to compare differences between groups. Myography results from isolated MCA and FEM were analysed using a 2-way repeated measures ANOVA and Tukey post hoc test was used to compare between groups or doses. Normality was

calculated using D'Agostino and Pearson and Kolmogorov-Smirnov normality test (Prism 7 for Mac OS X, GraphPad Software).

## Results

### Plasma Sildenafil Concentrations

Maternal intravenous infusion of SC resulted in detectable levels of SC within fetal (3.05±1.50 pg/μL) and maternal (16.72±3.56 pg/μL) plasma (Table). SC was undetectable in untreated animals (data not shown).

### Fetal Birthweight and Brain: Body Weight

Comparisons were made between saline-treated AG (n=7) and FGR (n=6) fetuses and SC-treated AG<sub>SC</sub> (n=5) and FGR<sub>SC</sub> (n=6) fetuses, Table. One FGR and 1 AG<sub>SC</sub> fetus were not included in analysis because of complications after surgery. Fetal birthweight shows SUAL resulted in a 25% reduction in birth weight compared with AG (*P*=0.03). Surprisingly, treatment with SC exacerbated FGR resulting in a ≈50% reduction in birth weight compared with AG (*P*<0.001). No significant difference in birthweight was seen between saline-treated and AG<sub>SC</sub> fetuses. Brain weight was similar between saline-treated and AG<sub>SC</sub> fetuses but significantly lower in FGR<sub>SC</sub> compared with AG control. Brain/body weight ratio was significantly increased in FGR<sub>SC</sub> compared with AG, *P*=0.003 and AG<sub>SC</sub>, *P*=0.0024. No other significant differences in brain/body weight were seen between groups.

### Myography Analysis

#### Arterial Contractile Ability

Maximal contraction to high potassium solution (0.6M) was not different in the MCA between any groups (Figure 1A). In contrast, in the FEM maximal contractile force was significantly reduced (*P*<0.05) in FGR, FGR<sub>SC</sub>, and AG<sub>SC</sub> groups compared with AG fetuses (Figure 1B). Contractile force within the FEM was not different between FGR, AG<sub>SC</sub>, and FGR<sub>SC</sub>.

#### Vasodilation in Response to SNP and Acetylcholine in MCA and FEM

SNP induces NO-mediated, endothelium-independent vasodilation. In the MCA, no difference in SNP-mediated vasodilation was seen between groups of the same treatment (AG versus FGR or AG<sub>SC</sub> versus FGR<sub>SC</sub>). Respective increasing vasodilation occurred from increasing doses of SNP (10<sup>-10</sup>–10<sup>-4</sup> mol/L) in all groups, and final SNP dose (10<sup>-10</sup> mol/L) resulted in greater vasodilation compared to initial SNP dose (10<sup>-4</sup> mol/L). MCA of both SC-treated groups vasodilated to lower concentrations of SNP compared with saline-treated. MCA vasodilation was significantly greater between SNP concentrations of 10<sup>-7</sup> to 10<sup>-5.5</sup> mol/L (Figure 2A; *P*<0.05) of SC compared with control. Increased overall vasodilatory response to SNP (smaller area under the curve) was observed in MCA of SC treated compared with saline (Figure 3A; *P*<0.05). Overall vasodilation to SNP was not seen within groups.

Acetylcholine mediates ligand-activated endothelium-dependent vasodilation. In MCA, there was no difference in vasodilation to acetylcholine between groups of the

**Table. Characteristics of Fetuses at Postmortem Delivery of AG, FGR, AG<sub>sc</sub>, and FGR<sub>sc</sub>**

	AG	FGR	AG <sub>sc</sub>	FGR <sub>sc</sub>
N	7	6	5	6
Sex (male)	6/7	3/6	2/5	2/6
Weight, kg	3.7±0.31	2.7±0.72*	2.8±0.46	1.9±0.48*
Brain weight, g	50.37±1.71	49.01±5.09	45.66±2.55	40.01±2.55*
Brain: body weight, g/kg	14.0±1.0	18.56±4.27	16.64±2.67	22.61±4.89*
	Fetal		Maternal	
Plasma sildenafil concentration, pg/μL	3.05± 1.50		16.72± 3.56	

Weights and SC concentration are mean±SD. Data were compared using a 2-way ANOVA to determine difference between treatments. Multiple comparisons were performed via a Tukey test. AG indicates appropriately grown; AG<sub>sc</sub>, SC-treated appropriately grown; FGR, fetal growth restriction; FGR<sub>sc</sub>, SC-treated fetal growth-restricted; and SC, sildenafil citrate.

\*Signifies significant difference ( $P<0.05$ ) compared with AG.

same treatment (AG versus FGR or AG<sub>sc</sub> versus FGR<sub>sc</sub>). Vasodilation occurred from increasing doses of acetylcholine ( $10^{-10}$  to  $10^{-4}$  mol/L) in both saline-treated groups, and final acetylcholine dose ( $10^{-10}$  mol/L) resulted in greater vasodilation compared with initial SNP dose ( $10^{-4}$  mol/L). Vasodilatory response was significantly greater at acetylcholine concentrations  $10^{-6}$  mol/L and  $10^{-4}$  mol/L of both saline-treated compared with SC-treated groups (AG<sub>sc</sub> and FGR<sub>sc</sub>, Figure 2B;  $P<0.05$ ). In contrast, vasodilation of SC treated was not different between any acetylcholine dose. Overall vasodilatory response to acetylcholine (smaller area under the curve) was greater in MCA of saline-treated compared with SC treated (Figure 3B;  $P<0.05$ ). Overall differences in vasodilation to acetylcholine was not seen within groups.

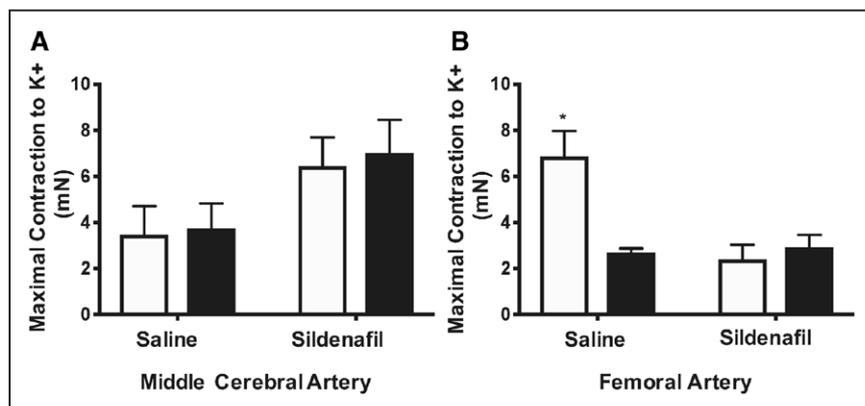
SNP-mediated vasodilation of FEM occurred from increasing doses of SNP ( $10^{-10}$  to  $10^{-4}$  mol/L) in of AG, FGR, and AG<sub>sc</sub>, and final SNP dose ( $10^{-10}$  mol/L) resulted in greater vasodilation compared with initial SNP dose ( $10^{-4}$  mol/L; Figure 2C,  $P<0.001$ ,  $P<0.001$ , and  $P=0.0074$ , respectively). Vasodilation in FEM of FGR<sub>sc</sub> was not different between starting and final SNP doses. Femoral response to SNP was significantly greater in saline-treated groups compared with SC-treated groups between SNP doses  $10^{-8}$  mol/L to  $10^{-4}$  mol/L (Figure 2C;  $P<0.05$ ). Increased overall vasodilatory response to SNP (smaller area under the curve) was observed in FEM of saline-treated compared with SC

(Figure 3C;  $P<0.05$ ). FEM of FGR saline controls had greater overall vasodilation compared with AG. (Figure 3C;  $P<0.05$ ). No difference in overall vasodilation to SNP was seen in SC treated.

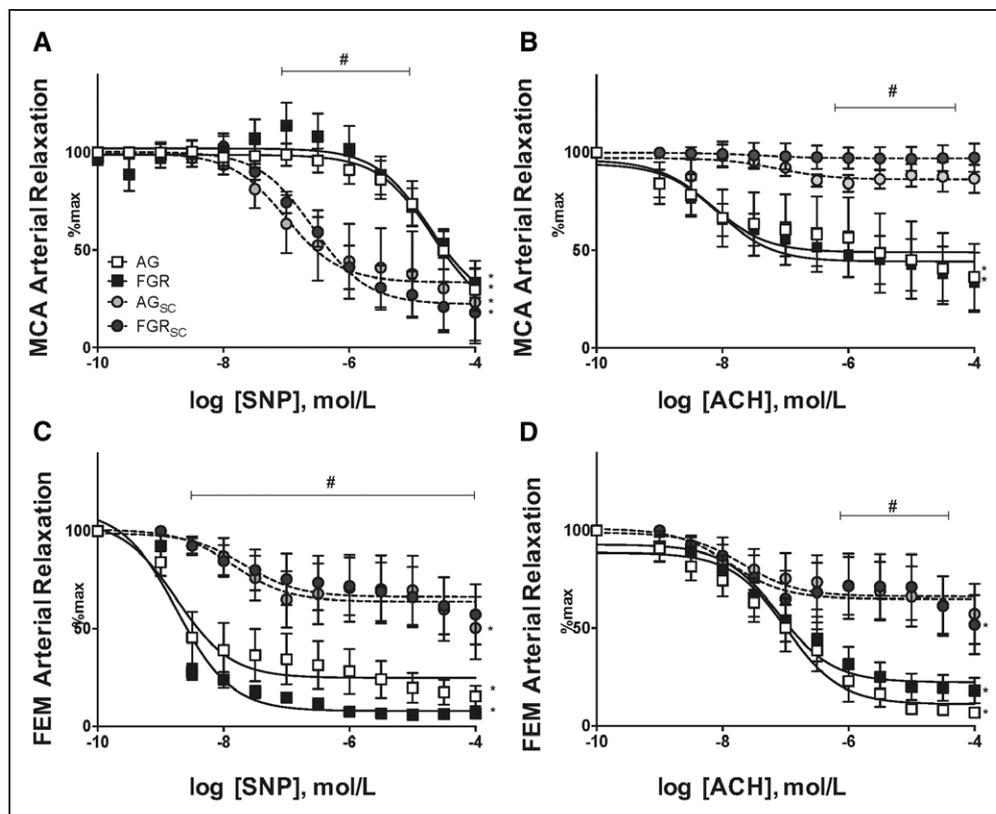
Acetylcholine-mediated vasodilation of FEM was not different within groups of the same treatment (AG versus FGR or AG<sub>sc</sub> versus FGR<sub>sc</sub>). Vasodilation in FEM from increasing doses of acetylcholine ( $10^{-10}$  to  $10^{-4}$  mol/L) occurred in AG, FGR and FGR<sub>sc</sub> and final acetylcholine dose ( $10^{-10}$  mol/L) resulted in greater femoral vasodilation compared with initial acetylcholine dose ( $10^{-4}$  mol/L; Figure 2D,  $P<0.001$ ,  $P<0.001$ , and  $P=0.0258$ , respectively). Vasodilation in FEM of AG<sub>sc</sub> was not different between starting and final acetylcholine doses. Vasodilation was significantly greater in saline-treated groups compared with SC-treated groups. This difference was significant between acetylcholine concentrations  $10^{-6}$  mol/L to  $10^{-5}$  mol/L (Figure 2D;  $P<0.05$ ). Significantly greater overall vasodilation (smaller area under the curve) occurred in saline-treated compared with saline (Figure 3D;  $P<0.05$ ). Overall differences in vasodilation to acetylcholine was not seen within groups.

#### Vascular Sensitivity and %Rmax of MCA and FEM in Response to SNP and Acetylcholine

SC-treated MCA had a greater sensitivity to SNP as shown by a significantly greater pEC<sub>50</sub> compared with saline-treated (Figure 4A;  $P<0.001$ ). %Rmax was greater in control lambs compared with SC treated (Figure 4A;  $P=0.0169$ ). MCA sensitivity to acetylcholine (pEC<sub>50</sub>) was not different between



**Figure 1.** Middle cerebral artery (MCA) and femoral artery (FEM) response high K<sup>+</sup> physiological saline solution (0.1M). Data are mean±SEM contractile force (mN) response of MCA (A) and FEM (B) in appropriately grown (AG, white, n=7), fetal growth-restricted (FGR, black, n=6), sildenafil citrate-treated AG (white, n=5) and sildenafil citrate-treated FGR (black, n=6) fetuses. Data were compared using a 2-way ANOVA to determine difference between treatments. \*Signifies significant difference ( $P<0.05$ ). Multiple comparisons were performed via a Tukey test.



**Figure 2.** Middle cerebral artery (MCA) and femoral artery (FEM) response to sodium nitroprusside (SNP) and acetylcholine (ACh). Data are mean±SEM vasodilation from maximal contraction (% max) of MCA in response to SNP and ACh (A and B, respectively) and FEM in response to SNP and ACh (C and D, respectively) of appropriately grown (AG, white, n=7), fetal growth-restricted (FGR, black, n=6), sildenafil citrate (SC)-treated AG (gray, n=5) and SC-treated FGR (dark gray, n=6). Data were compared using a 2-way ANOVA to analyze myography results between treatment groups. #Signify a significant difference in vasodilation between saline and SC-treated groups and \*signify a significant dose difference compared with 10<sup>-10</sup> mol/L within group.

groups (Figure 4B). %Rmax was significantly impaired in AG<sub>SC</sub> and FGR<sub>SC</sub> vessels compared with saline-treated groups. %Rmax was significantly lower in AG<sub>SC</sub> and FGR<sub>SC</sub> compared with saline-treated (Figure 4B, P<0.001). No difference in pEC50 and %Rmax was seen within treatment groups.

Femoral vascular sensitivity (pEC<sub>50</sub>) to SNP was significantly lower in SC-treated lambs compared with control (Figure 4C; P=0.0255). %Rmax was significantly greater in control lambs compared with SC treated (Figure 4C; P>0.001). Femoral vascular sensitivity (pEC<sub>50</sub>) to acetylcholine was not different between groups. Femoral %Rmax to acetylcholine (%Rmax) was significantly greater in control lambs compared with SC treated (Figure 4D; P>0.001). No difference in pEC50 and %Rmax was seen within treatment groups.

**Contribution of cGMP to Relaxation**

To determine the relative contribution of cGMP to overall relaxation, PDE5 inhibitor SC was used. These data reveal a significantly greater cGMP capacity of sildenafil-treated fetuses compared with saline-treated fetuses (P=0.0059; Figure 5).

**Protein Levels of Soluble Guanylate Cyclase**

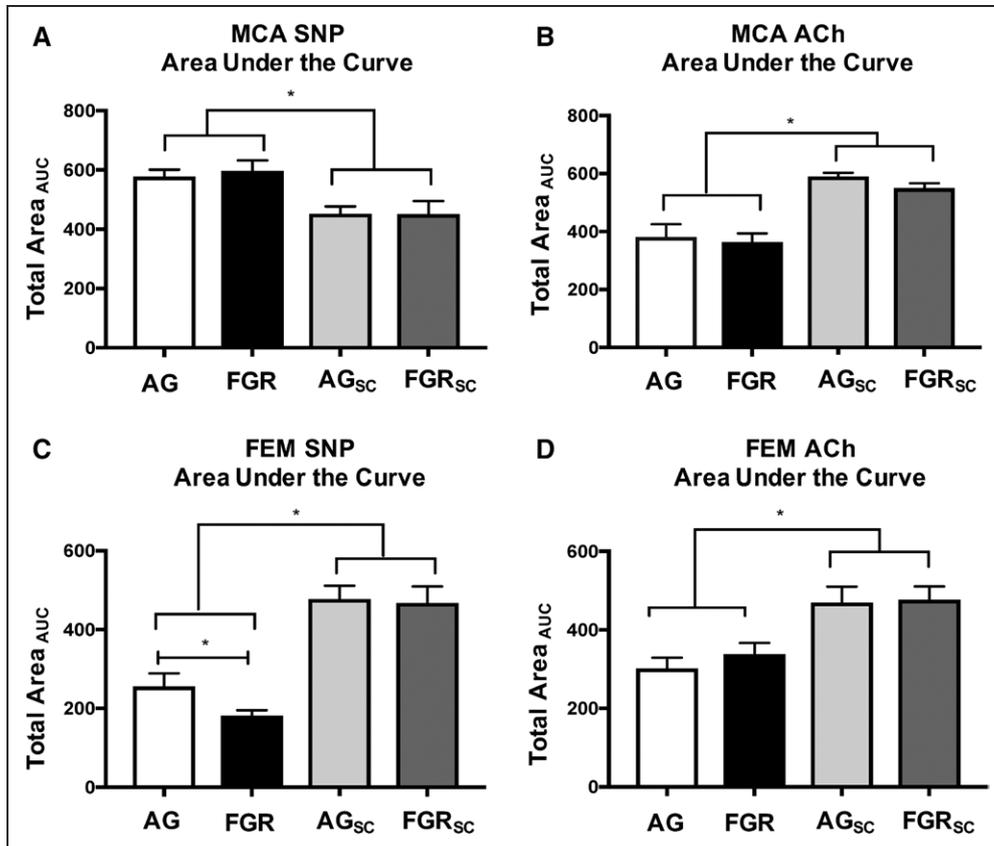
Protein levels of sGC within the FEM were not different between groups (data not shown).

**Discussion**

FGR increases the risks of long-term dysfunctions.<sup>27</sup> There is no cure for FGR; however, maternal administration of SC has

suggested improvement in some animal studies of FGR<sup>28–30</sup> and in small human trials.<sup>12,31</sup> In turn, SC has been the subject of the worldwide STRIDER clinical trial as a potential therapy for FGR. However, reports suggest that antenatal SC may increase mortality for the growth-restricted infant after birth.<sup>15</sup> There is currently limited data to show effects of SC on the already compromised cardiovascular structure and function in the setting of FGR. Assessment of vascular contractile ability is indicative of vascular health, integrity and can inform of potentially dysfunctional vascular pathways.<sup>32</sup> In the current study, we have validated the transfer of SC from the maternal circulation into the fetal circulation. Furthermore, we show that maternal SC administration exacerbated growth restriction and results in vascular dysfunction in the developing peripheral and cerebral cardiovascular system of FGR and AG fetuses. These differences are important. It has long been demonstrated that homeostasis within the peripheral vascular system is vital for blood pressure control. Of equal concern is dysfunction in the cerebral circulation, wherein exposure to SC caused endothelial dysfunction of cerebral vessels in FGR fetuses, which has implications for the brain-sparing adaptation mounted by the growth-restricted fetus, and acute cerebral responses to challenges after birth.

SC has been used clinically for many years. SC is a PDE 5 inhibitor with various applications, including erectile dysfunction (Viagra) and persistent pulmonary hypertension of the newborn.<sup>33</sup> SC induces vasodilation through the



**Figure 3.** Area under the curve (AUC) of middle cerebral artery (MCA) and femoral artery (FEM) response to sodium nitroprusside (SNP) and acetylcholine (ACh). Data are mean±SD of AUC from MCA in response to SNP and ACh (A and B, respectively) and FEM in response to SNP and ACh (C and D, respectively), of appropriately grown (AG, white, n=7), fetal growth-restricted (FGR, black, n=6), SC-treated AG (AG<sub>SC</sub>, gray, n=5) and SC-treated FGR (FGR<sub>SC</sub>, dark gray, n=6). Data were compared using a 2-way ANOVA. \*Signify significant difference between treatments (P<0.05).

NO-cyclic cGMP pathway. PDE5 regulates intracellular levels of cGMP by hydrolyzing cGMP to GMP. Modulation of cGMP levels is critical for basal vascular tone and reactivity, where PDE5 promotes increased breakdown of cGMP and, therefore, vasoconstriction. SC inhibition of PDE5 thus promotes vasodilation.

Animal and human studies have shown that SC crosses the placenta to enter the fetal circulation.<sup>16</sup> Itani et al have demonstrated protective cardiovascular effect of SC in hypoxic chicken embryos.<sup>34</sup> Although this study did not demonstrate an increase in fetal growth, SC treatment prevented oxidative stress, enhanced NO bioavailability, and restored peripheral endothelial function.<sup>34</sup> Our study is the first to demonstrate SC crosses the ovine placenta. SC concentrations of 3.5 nmol/L is considered clinically relevant and has the ability to inhibit 50% of PDE5 activity.<sup>35</sup> The difference in fetal to maternal SC concentrations is likely because of limitations in ovine placental transfer. However, the dose given to ewes resulted in fetal SC concentrations sufficient to inhibit >50% of fetal PDE5 activity.

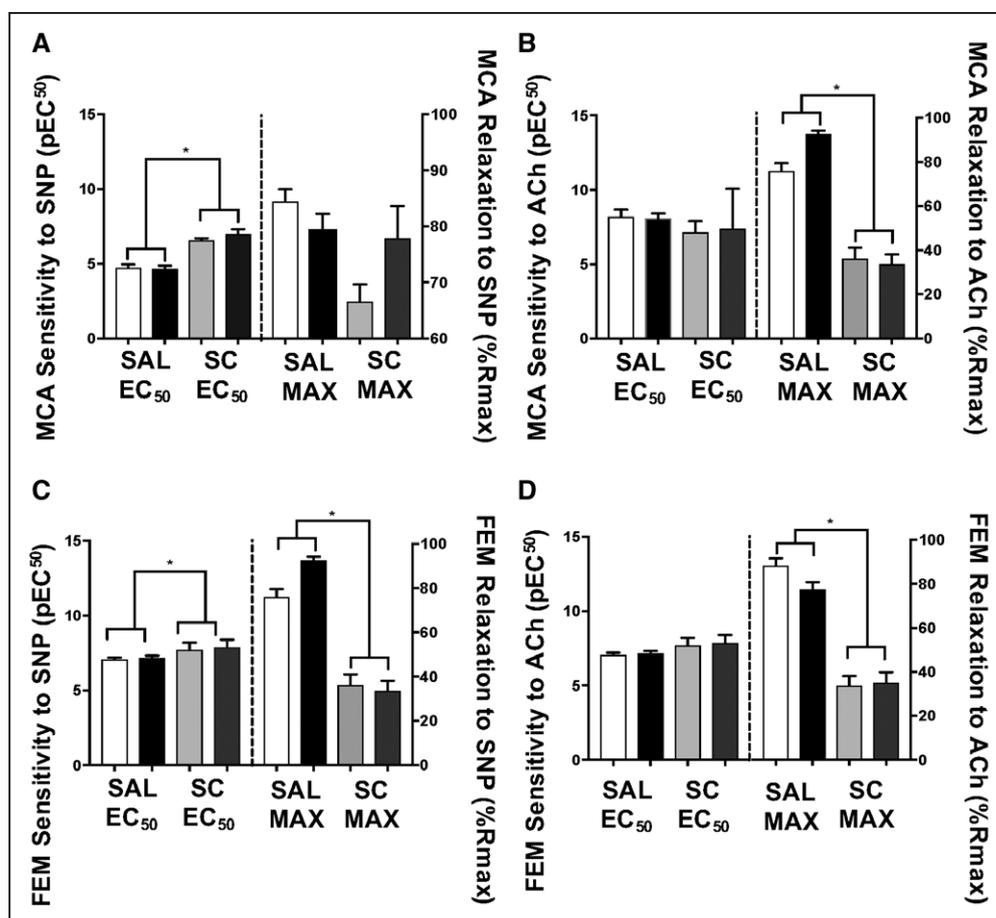
### Exacerbation of FGR

An unexpected but striking observation of this study is the exacerbation of FGR in fetal sheep after SC treatment. In this study, as in previous studies, SUAL resulted in asymmetrical growth restriction.<sup>19</sup> Antenatal SC exacerbated body weight deficits in FGR, resulting in a 49% reduction in FGR<sub>SC</sub> compared with

AG fetuses, a finding accompanied by brain-sparing in FGR<sub>SC</sub> fetuses. No other studies have reported such striking decreases in body weight, but not all studies describe improved body weight. Although rodent studies have demonstrated increased fetal body weight,<sup>13</sup> other human and sheep studies do not show improvement in body weight.<sup>36,37</sup> The mechanisms underlying increased growth restriction were not interrogated in this study, but it is likely that maternal hemodynamics play a central role. Miller et al<sup>38</sup> previously found maternal SC administration to ovine pregnancies complicated by FGR had the potential to cause detrimental uteroplacental flow. This study also reported fetal hypotension and tachycardia after maternal SC treatment.<sup>38</sup>

It is important to note that although decrease in body weight in AG<sub>SC</sub> compared with AG lambs was not significant, body weights were ≈900g lighter and similar to that of FGR. We speculate that the reduction in body weight observed in both SC-treated groups may have occurred in either 1 or a combination of 2 ways:

The proposed mechanism of benefit of SC is through the increase in vasodilation of the placental vasculature and subsequent increase in blood flow. As previously discussed, our model recreates FGR through SUAL, which results in subsequent atrophy to half the placental surface area.<sup>39</sup> In our study, we think that any increase in blood flow by SC could only supply the still functional placenta regions. It is unknown if SC has the ability to



**Figure 4.** Half-maximal effective concentration ( $pEC_{50}$ ) and total maximal dilation percentage (%Rmax) of middle cerebral artery (MCA) and femoral artery (FEM) response to sodium nitroprusside (SNP) and acetylcholine (ACh). Data are mean $\pm$ SEM vasodilation of  $pEC_{50}$  and %Rmax of MCA in response to SNP and ACh (A and B, respectively) and FEM in response to SNP and ACh (C and D, respectively) of appropriately grown (AG, white, n=7), fetal growth-restricted (FGR, black, n=6), sildenafil citrate-treated AG (AG<sub>sc</sub>, gray, n=5) and sildenafil citrate-treated FGR (dark gray, n=6). Data were compared using a 2-way ANOVA. \*Signify significant difference between treatments ( $P < 0.05$ ). SAL indicates saline.

repair malformed blood vessels and, therefore, in the context of impaired spiral artery remodeling, should similar placental vasodilation occur clinically, benefit of SC will also be limited. Given the observed reduction in fetal size after SC treatment seen in this study, we think that caution surrounding SC treatment is warranted.

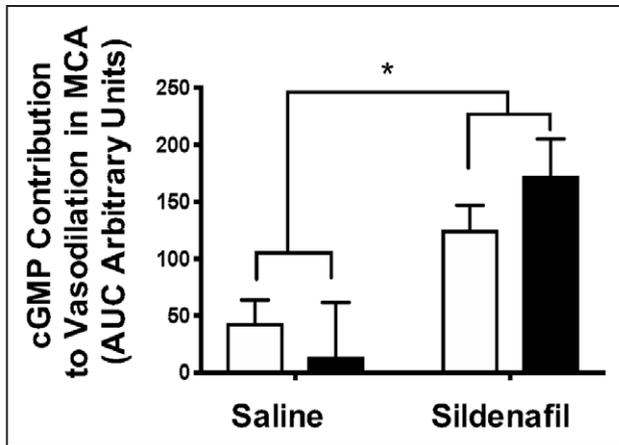
It is possible that long-term maternal SC administration may lead to chronic peripheral vasodilation within the maternal circulation, resulting in shunting of blood away from the fetoplacental circulation, further diminishing placental perfusion. Though not assessed in the current study, the increased abnormality in ductus venosus a-wave (UK STRIDER) may support our finding.<sup>36</sup> Reversal of flow through the ductus venosus may occur because of a decrease in pressure driven by vasodilation of the maternal and placental circulation.

**Vascular Effects of FGR and SC**

To interrogate vascular function, we measured vascular ability to constrict to K<sup>+</sup>, endothelial-mediated vasodilation using acetylcholine-mediated and smooth muscle-mediated vasodilation using SNP. We chose to investigate central and peripheral vascular beds because these are believed to be spared (MCA-central) and nonspared (femoral-peripheral)

from the vascular consequences of FGR. FGR-associated endothelial dysfunction in the periphery is well described and found to be NO dependent,<sup>9</sup> with restoration possible via treatment with antioxidants such as vitamin C<sup>40</sup> and melatonin.<sup>41</sup> Additionally, Itani et al<sup>41</sup> show cardiovascular protection in chicken embryos after SC, suggesting potential benefit of SC treatment. FGR is associated with heterogeneous vascular remodeling in FGR offspring, where central vessels are favored or less affected compared with the vasculature of peripheral organs.<sup>42</sup> Our findings also support this study as FGR alone did not alter vascular responses in the cerebral circulation.

Sildenafil treatment impaired endothelial-mediated vasodilation in the MCA but enhanced the smooth muscle mediated vasodilation. The difference in response may be because of potential compensatory mechanisms in the NO pathway of the MCA smooth muscle after SC exposure. Increased sensitivity to SNP after SC treatment is indicative of developmental resetting of the NO pathway. This compensation was not observed in the peripheral circulation. Renshall et al<sup>43</sup> have also previously shown impaired endothelium-dependent and endothelium-independent vasodilation in SC-treated FGR mouse aorta, considered to be a central vascular bed. As both



**Figure 5.** cGMP contribution to vasodilation of the middle cerebral artery (MCA). Data are mean±SEM area under the curve (AUC) of vasodilation of the MCA in appropriately grown (AG, white, n=7), fetal growth-restricted (FGR, black, n=6), sildenafil citrate-treated AG (n=5) and sildenafil citrate-treated FGR (n=6) fetuses. Data were compared using a 2-way ANOVA to determine difference between treatments and growth status. \*Signifies significant difference ( $P<0.05$ ). Multiple comparisons were performed via a Tukey test.

direct administration of NO to the smooth muscle and inhibition of cGMP degrader, PDE5, resulted in enhanced vasodilation, it is clear that the vascular smooth muscle is intact. In contrast, endothelial-dependent vasodilation is nearly entirely attenuated; thus, SC administration is having its effects within the endothelium, likely because of decreased NO bioavailability or altered agonist receptor population. The potential cerebrovascular dysfunction may be described as a decrease in endothelial function and vascular reactivity observed in both FGR and AG following SC exposure. These results are concerning as they suggest exposure to SC in utero, rather than placental insufficiency, results in endothelial dysfunction. Interestingly, Kruse et al<sup>44</sup> found that MCA from adult rats do not vasodilate to SC administration.

The maintenance of cerebral blood flow in FGR is a well-described response in FGR<sup>45</sup>; further studies by Poudel et al<sup>46</sup> have shown that carotid blood flow between FGR and AG lambs is similar, despite increased systemic vascular resistance. We have furthered these findings to show that there is little difference in vasodilation of MCA between FGR and AG fetuses in our current study. Thus during brain-sparing, blood vessel function within spared beds is not altered.

However, despite attempts at maintaining cerebral perfusion, the FGR brain is susceptible to injury after FGR.<sup>19</sup> Given this, the diminished functionality of the cerebral vasculature following SC exposure is concerning, considering impaired autoregulation is associated with an increased risk of brain injury.<sup>47</sup> Further studies to interrogate mechanism on how antenatal SC exposure alters cerebrovascular development, and potential long-term effects, would provide greater insight to chronic SC exposure during pregnancy.

Although in the dysfunction in the cerebral vasculature are endothelial in origin, in the femoral vascular bed, SC administration decreased the vasodilatory response of the vascular smooth muscle to SNP, a NO donor that binds to sGC to cause vascular relaxation. Vascular smooth muscle relaxation

occurs as a result of decreased intracellular calcium, and there are numerous pathways, such as the adenylate cyclase-cGMP pathway, which also play a role in vasodilation. In the current study, we focused on activation of sGC, but it is also possible that other pathways may be upregulated following antenatal treatment with SC. We observed that SC treatment impaired fetal peripheral smooth muscle function resulting in endothelial dysfunction. Although the peripheral vascular effects may result from fetal exposure to SC per se, it is also possible that a worsening of growth restriction may contribute to peripheral vascular deficits. Endothelial dysfunction is a well-established consequence of FGR,<sup>41</sup> the exacerbation of FGR observed in our study is of particular importance to vascular function.

PDE5 inhibition has been associated with smooth muscle preservation<sup>48</sup> and may explain the increased sensitivity to SNP observed in MCA. However, in contrast drastically lower maximal constriction was observed in FGR peripheral vessels. These differences are likely because of the hemodynamic adaptations mounted during brain-sparing in FGR. Unexpectedly, similar responses were seen within the femoral of both SC-treated groups, regardless of growth status. These findings are concerning as they suggest that independent SC exposure during development has the ability to disrupt smooth muscle and K<sup>+</sup> channel activity. Studies investigating SC actions on pulmonary hypertension have shown that SC exposure reduces smooth muscle deposition<sup>49</sup> and has antiproliferative properties within the smooth muscle via the eNOS-NO-cGMP pathway.<sup>50</sup>

The pathway of NO-mediated vasodilation within the smooth muscle involves several key mediators. cGMP causes smooth muscle relaxation, proliferation, and differentiation<sup>51</sup> and is broken down by PDE5, the target of SC action. We and others have shown that SC exposure increases cGMP levels.<sup>52</sup> Of particular importance to this study, increased levels of cGMP and cGMP elevating agents can suppress proliferation of smooth muscle.<sup>53</sup> The observed decrease in smooth muscle response seen in SC-treated fetuses supports the potential for cGMP to disrupt smooth muscle proliferation. Furthermore, although SC exposure increased cGMP contribution to vasodilation, overall dilation was reduced in SC-treated fetuses (Figure 5B). These data together support the hypothesis that SC treatment in utero reduces NO bioavailability. cGMP curves were only conducted in MCA vessels, and therefore it is not possible to extrapolate this data to the FEM.

### Limitations

In this study, we aimed to mimic the human dose of SC administration given in the STRIDER trials. To provide a controlled administration of SC to the pregnant ewe, SC was given intravenously to the ewe. This is in contrast to the STRIDER trial in which SC was taken orally in 3 tablets over the course of the day. Thus, the exposure to SC is likely to be different between our study and the human trial. SC taken orally has a bioavailability of ≈41 %<sup>54</sup>; thus in the current study, we gave a dose of 36 mg per day to replicate this level. Furthermore, we commenced SC treatment at 3 days after inducing placental insufficiency, which is rather early compared to the human situation in which placental insufficiency and chronic hypoxia are likely to be well progressed. The delay in response to SNP,

relative to that of acetylcholine within the MCA of control animals, may suggest alteration of MCA smooth muscle from our SUAL intervention. Future studies should aim at interrogating the NO-mediated signaling pathways in this vessel. We also do not have measurements of uterine flow in this study and future studies should aim at investigating the effect of long-term SC treatment on uterine flow to better allude to the increased FGR observed. Additionally, because of the low numbers used in these studies, it was not possible to separately analyze the effects of fetal sex.

**Conclusions**

Although previous animal studies have shown SC to improve growth in FGR, the current study did not support those findings. Rather, SC resulted in exacerbation of growth restriction and additionally was detrimental to vascular function. Cerebral vascular function is normally preserved in growth-restricted offspring due to brain-sparing, but here we show that antenatal SC exposure impaired vascular function in this vital vascular bed. In turn, this has significant implications for the infant after birth, potentially limiting cerebral vascular reactivity to acute challenge and increasing the risk of neurological compromise. This study shows specific effects of SC exposure on the developing cardiovascular system of the fetus, which may, in turn, have longer-term detrimental effects on cardiovascular health. SC crosses the ovine and human placenta and accordingly, we suggest that the safety of SC should be considered with long-term cardiovascular health of the offspring in mind.

**Acknowledgments**

We gratefully acknowledge the technical assistance of Ilias Nitsos, Jade Barbutto, Valerie Zahra, and Alison Moxham.

**Sources of Funding**

This study was supported by NHMRC (1083520) and Cerebral Palsy Alliance Project Grant PG0414.

**Disclosures**

None.

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### Highlights

- Maternal sildenafil citrate exacerbated growth restriction in an ovine model of fetal growth restricted.
- In utero exposure to sildenafil citrate increased cerebral smooth muscle reactivity to NO.
- In utero exposure to sildenafil citrate impaired endothelial function in the cerebral and peripheral vasculature.
- Fetal exposure to sildenafil citrate may have unknown adverse effects and should be extensively studied to determine impact on the clinical setting.