Maternal Administration of Sildenafil Citrate Alters Fetal and Placental Growth and Fetal–Placental Vascular Resistance in the Growth-Restricted Ovine Fetus

Charlotte Oyston, Joanna L. Stanley, Mark H. Oliver, Frank H. Bloomfield, Philip N. Baker

Abstract—Intrauterine growth restriction (IUGR) causes short- and long-term morbidity. Reduced placental perfusion is an important pathogenic component of IUGR; substances that enhance vasodilation in the uterine circulation, such as sildenafil citrate (sildenafil), may improve placental blood flow and fetal growth. This study aimed to examine the effects of sildenafil in the growth-restricted ovine fetus. Ewes carrying singleton pregnancies underwent insertion of vascular catheters, and then, they were randomized to receive uterine artery embolization (IUGR) or to a control group. Ewes in the IUGR group received a daily infusion of sildenafil (IUGR+SC; n=10) or vehicle (IUGR+V; n=8) for 21 days. The control group received no treatment (n=9). Umbilical artery blood flow was measured using Doppler ultrasound and the resistive index (RI) calculated. Fetal weight, biometry, and placental weight were obtained at postmortem after treatment completion. Umbilical artery RI in IUGR+V fell less than in controls; the RI of IUGR+SC was intermediate to that of the other 2 groups (mean±SEM for control versus IUGR+V versus IUGR+SC: ∆RI, 0.09±0.03 versus −0.01±0.02 versus 0.03±0.02; F(2, 22)=4.21; P=0.03). Compared with controls, lamb and placental weights were reduced in IUGR+V but not in IUGR+SC (control versus IUGR+V versus IUGR+SC: fetal weight, 4381±247 versus 3447±235 versus 475.2±42.5 g; F(2, 24)=4.64; P=0.01). Sildenafil may be a useful adjunct in the management of IUGR. An increase in placental weight and fall in fetal–placental resistance suggests that changes to growth are at least partly mediated by changes to placental growth rather than alterations in placental efficiency. (Hypertension. 2016;68:760-767. DOI: 10.1161/HYPERTENSIONAHA.116.07662.) • Online Data Supplement

Key Words: fetal development ■ placental circulation ■ sildenafil citrate ■ ultrasonography

Intrauterine growth restriction (IUGR) occurs when a fetus fails to achieve its genetic growth potential. These fetuses have an increased risk of significant perinatal morbidity and mortality,1,2 neurodevelopmental impairment in childhood,3 and an increased risk of adult onset chronic diseases.4 Currently, there are no clinically available treatments that can improve fetal growth. The management of IUGR pregnancies is limited to intense fetal monitoring in an attempt to determine when the fetus has maximized its time in utero and when the risks of hypoxia and death are so high that early delivery is indicated. When IUGR is severe or of early onset, delivery may be indicated at extremely preterm gestations when the risk of perinatal complications are especially high.5 Of infants with birth weight <10th centile, those delivered at 30 to 32 weeks have a more than 3-fold greater chance of survival free of major morbidity, compared with those delivered at 24 to 28 weeks.6 A therapy facilitating expectant management and safe pregnancy prolongation has the potential to reduce dramatically both short- and long-term health and societal costs.

There are many causes of IUGR; however, the predominant cause is placental insufficiency, caused by a failure of placental trophoblast to adequately invade and transform maternal spiral arteries in early pregnancy.6,7 The net result of this abnormal transformation is increased resistance to maternal blood flow to the placenta resulting in placental underperfusion.8 In normal pregnancy, the vasodilator nitric oxide contributes to the increased vasodilation and reduced vascular resistance seen in the uteroplacental circulation.9,10 The nitric oxide second messenger cGMP is enzymatically degraded by phosphodiesterases. Sildenafil citrate (sildenafil), an inhibitor of phosphodiesterase-5 (an isoform found extensively throughout the reproductive tract11,12), is able to enhance the vasodilatory action of nitric oxide. Thus, it is hypothesized that sildenafil will improve uterine and placental perfusion in compromised pregnancies, increasing placental exchange and fetal growth. This hypothesis is supported by ex vivo studies of myometrial resistance arteries from growth-restricted pregnancies, which showed reduced
constriction and enhanced relaxation after preincubation with sildenafil.14

There is accumulating evidence of efficacy of sildenafil at improving fetal growth, particularly in rodents15-16; however, evidence of an effect of sildenafil treatment on fetal growth in larger species is limited.18 It is important to study efficacy in larger species, as duration of pregnancy (and therefore timeframe for treatment) and organ development are more akin to that seen in humans, thereby increasing the potential for identifying detrimental effects, as well as any beneficial effects. The current study was designed to investigate whether maternal administration of sildenafil citrate improves growth of lambs where growth restriction had been induced by uterine artery embolization and to explore potential mechanisms underlying any improvement in fetal growth.

Methods and Materials

Ethics Statement

All experimentation was conducted in accordance with accepted standards of humane care, with all experiments approved by the University of Auckland Animal Ethics Committee (approval number, AEC 001101).

Animals

Time-mated multiparous Romney-cross ewes carrying singleton pregnancies were acclimatized to indoor individual pens. Ewes were randomized preoperatively to a control group (no uterine artery embolization) or an IUGR group that received embolization. Surgery was performed between 96 and 100 days of gestational age (dGA, term=147 dGA). After an overnight fast, general anesthesia was induced with 30 mL IV propofol and maintained with inhaled isoflurane. As previously described,20 a midline laparotomy and hysterotomy were performed, and polyvinyl catheters were placed into both fetal femoral arteries and veins via the tarsal vessels. A single free-floating amniotic fluid catheter was inserted before closing the hysterotomy. Maternal uterine veins were catheterized bilaterally with polyvinyl catheters with silicone tips. In the IUGR groups, the main uterine arteries were catheterized via a distal arterial branch. After closure of the maternal abdomen, a maternal femoral artery and vein were catheterized via the tarsal vessels. The ewe received a single intramuscular dose of 450 IU benzathine penicillin, and the fetus received 80 mg gentamicin sulfate: Yellow Springs Instruments, Dayton, OH). Fetal samples were collected for the first 3 days postoperatively, then twice daily during the embolization period, and then twice weekly for the remaining duration of the experiment.

Uterine Artery Embolization

From 102 to 107 dGA, growth restriction was induced by up to twice daily embolization of the uterine arteries with polystyrene microspheres of 20- to 50-µm diameter (Supercord 12, 1:100 dilution; Pharmacia Biotech, Uppasala, Sweden) as described previously.20 The frequency and volume of injections were titrated against fetal PaO2 and lactate levels, with embolization withheld if fetal PaO2 was <14 mmHg or fetal arterial lactate was >4 mmol/L.

Experimental Period

After completion of embolization, ewes in the IUGR group were randomized to treatment with sildenafil citrate (150 mg per day; Zhuhai Jiacheng Bio-Tech, Zhuhai City, China) dissolved in 54 mL sterile water (IUGR+SC group) or a visually indistinguishable infusion of 54 mL vehicle sterile water (IUGR+V group). As there are no published data on the pharmacokinetics of sildenafil citrate in the pregnant ewe, the dose of sildenafil was chosen in line with previous studies where biological effect of sildenafil was apparent.19 Sildenafil was administered via a continuous subcutaneous infusion over 12 hours via a portable infusion pump secured to the ewe’s back (WalkMed Infusion, Centennial, CO). The infusion bag of the pump was checked and refilled at the same time each day, and any residual fluid was given as a slow subcutaneous bolus. The infusion site, subcutaneous needle, and infusion tubing were changed every third day to minimize the risk of infection and localized pooling of the infusate.

Assessment of Umbilical and Uterine Artery Blood Flows

Ultrasound assessment of umbilical artery blood flow was performed before surgery (96 dGA) and at 107, 119, and 128 dGA. With the ewe nonsedated and standing, a free-floating segment of umbilical cord was identified with pulsed wave color Doppler. Waveforms were recorded during fetal quiescence with the angle of insonation of <50°. Recorded images were reviewed offline, and umbilical artery resistance index was calculated using an average of 3 consecutive waveforms, using the following formula: resistive index (RI)= (S-D)/S, where S is the peak systolic velocity and D the height of the end diastolic trough. Ultrasound images were obtained and analyzed by an investigator blinded to treatment group for the IUGR animals.

At 107, 119, and 128 dGA, assessment of uterine artery blood flow was performed via infusion of antipyrine using the Fick principle. A tracer solution containing 160 mg of antipyrine in 20 mL of saline was infused into a fetal vein at 3 mL/h after a 4-hl bolus. Under these conditions, previous studies have shown that antipyrine reaches a steady state at 90 minutes.21 From 90 minutes, 4 sets of paired blood samples drawn from a maternal artery and the utero-ovarian vein were collected at 15-minute intervals. Antipyrine concentration was measured by High Performance Liquid Chromatography as described previously,22 with a Phenomenex Kinetex column (1.7 µm C18[2] 100 Å), dimensions 150×2.1 mm (Phenomenex, Torrance, CA). Uterine artery blood flow was then calculated using the antipyrine steady-state diffusion method with the application of the Fick principle.

Tissue Collection

At 132 to 133 dGA, ewes were euthanized with an overdose of intravenous pentobarbitone. The uterus was removed and opened. The fetus was removed, dried, weighed, and measured; fetal organs were dissected and weighed. Immediately after removing the fetus, a full-thickness uterine biopsy was taken from a site proximate to umbilical cord insertion, and myometrial resistance vessels were dissected clean and stored in ice cold physiological saline solution (NaCl, 136.9 mmol/L; KCl, 2.7 mmol/L; CaCl2, 1.8 mmol/L; MgSO4, 0.6 mmol/L; NaHCO3, 11.9 mmol/L; KH2PO4, 0.5 mmol/L; and glucose, 11 mmol/L; pH 7.4). The ruminant placenta is made up of many placentomes that develop at uterine attachments sites called caruncles. These placentomes can take a variety of morphological appearances and have been classified as A to D accordingly.23 Placentomes were dissected from the uterus, sorted into categories A, B, C, or D, counted, and weighed.

Ex Vivo Vascular Function

Segments of myometrial resistance vessels from each sheep were mounted on a wire myograph system (Multi Myograph System 610 M; Danish Myo Technology A/S, Aarhus, Denmark) and normalized within 8 hours of dissection, as previously described.24 Vessels were constricted with phenylephrine (10 µmol/L; Sigma-Aldrich, Auckland, New Zealand) to confirm viability, washed, and equilibrated with physiological saline solution for 20 minutes. A second dose of phenylephrine (10 µmol/L) was given, and constriction was allowed to plateau before giving a single dose of acetylcholine (10 µmol/L; Sigma-Aldrich) to confirm endothelial integrity. After a further washout and 30-minute equilibration
period, a concentration–response curve to phenylephrine was constructed (0.1–10 μmol/L). The EC₅₀ concentration was calculated for individual vessels and used to preconstrict the arteries to construct concentration–response curves to acetylcholine (0.1–10 μmol/L) and then sodium nitroprusside (0.1–10 μmol/L; Sigma-Aldrich). Finally, a 120 mmol/L potassium solution (potassium physiological saline solution; HEPEs, 10 mmol/L; NaCl, 24 mmol/L; KCl, 124 mmol/L; MgSO₄, 2.4 mmol/L; CaCl₂, 4.9 mmol/L; KH₂PO₄, 1.18 mmol/L; glucose, 5.5 mmol/L; pH, 7.4) was added to each vessel, and the constriction was allowed to plateau.

Data Analysis
Statistical analysis was performed using GraphPad Prism (version 6.03) software. Data are presented as mean±SEM or median (inter-quartile range) with significance determined by 1-way ANOVA and Tukey multiple comparisons test or by the Kruskall–Wallis test and Dunn multiple comparisons test as appropriate. For all analyses, a P value of <0.05 was considered statistically significant.

Fetal weights were normally distributed; histograms were constructed for each group, and nonlinear regression was performed to obtain Gaussian distributions. The fifth percentile of the control group fetal weight was calculated and used to define fetal growth restriction. Fetal arterial blood gas and glucose concentrations were compared between 2 groups (control versus IUGR) during embolization and before the onset of treatment (102–107 dGA) and then 3 groups (control versus IUGR+V versus IUGR+SC) after completion of embolization (110–129 dGA) using the appropriate nonparametric test.

For analysis of wire myography data, sigmoidal curve fitting was used to determine EC₅₀ for each substance.

Results
Forty-nine ewes underwent surgery (8 controls versus 41 IUGR). Of these, 20 were euthanized before the completion of the study: 14 because of perioperative fetal losses (IUGR group only), 4 fetal losses during embolization (IUGR group only), and 2 fetal losses occurred during the treatment period (one each from control and IUGR+SC group). The IUGR+SC lamb that died during the treatment period had extremely poor blood gasses post embolization (pH, 7.2; PaCO₂, 69 mmHg; PaO₂, 8.4 mmHg before commencing treatment) and died on the first day of treatment administration. Two ewes in the embolization group were excluded as embolization could not be adequately performed because of technical problems with laboratory equipment in the week of embolization. This left a total of 27 animals in the final analysis: 9 control, 8 IUGR+V, and 10 IUGR+SC. There were no differences among groups in maternal weight at the beginning of the experimental period or gestational age at surgery.

Fetal Growth
At postmortem, mean fetal weight was significantly different among groups (F(2, 24)=5.48; P=0.01). Mean fetal weight in the IUGR+V group was significantly less than controls (P=0.01); however, IUGR+SC–treated fetuses were not significantly different from controls or IUGR+V (control versus IUGR+V versus IUGR+SC: 4381±247 versus 3448±236 versus 3687±129 g; Figure 1A). The fifth centile of the control group was 3162 g; 38% of the IUGR+V lambs and 10% of the IUGR+SC group had a weight below this at postmortem (Figure 1B).

Fetal crown-rump length differed among groups (F(2, 24)=4.11; P=0.03), with lambs in the IUGR+V group significantly shorter than those in the control group (P=0.04). The crown-rump length of lambs in the IUGR+SC group did not differ from that of either of the other 2 groups (control versus IUGR+V versus IUGR+SC: 57.8±0.6 versus 45.1±1.0 versus 45.5±0.7 cm). There were no other significant differences in lamb measurements or organ weights between any of the groups when expressed as either absolute weight (Table 1) or as a proportion of body weight (data not shown).

Placental weight differed significantly among groups (F(2, 24)=5.52; P=0.01). Placental weight was significantly reduced in the IUGR+V group compared with controls (P<0.01), but in the IUGR+SC group, this was not significantly different to either of the other 2 groups (placental weight control versus IUGR+V versus IUGR+SC: 560±35 versus 376±33 versus 475±43 g; Table 2). The absolute number of placentomes differed between groups (F(2, 24)=5.22; P<0.01), with the IUGR+SC group having significantly more placentomes than IUGR+V (P<0.01), although neither group differed significantly from control (number of placentomes for control versus IUGR+V versus IUGR+SC: 70±5 versus 57±4 versus 80±5). Mean placental weight differed among groups (F(2, 24)=4.64; P=0.02) with placenta weight significantly reduced in the IUGR+SC group compared with controls (P<0.02) but not compared with IUGR+V (mean placenta weight for control versus IUGR+V versus IUGR+SC: 8.2±0.6 versus 6.8±0.7 versus 5.9±0.3 g). The IUGR+SC group had significantly lesser proportion of placentomes weighing ≥4 g compared with the control group (P<0.02; Table 2), but the proportion of large placentomes did not differ significantly from that in the controls.
All groups had similar proportions of A, B, and C placenta
types (Table 2). The IUGR+V group had significantly
fewer D-type placenta
tones compared with the control group
(P=0.01) but not the IUGR+SC group.

### Fetal Arterial Blood Gas and Glucose

Concentration

Blood sampling from fetal catheters became less reliable
with increasing gestation. Because of intermittent catheter

### Table 2.  Placenta
tome Weights and Morphology

<table>
<thead>
<tr>
<th>Measure</th>
<th>Control (n=9)</th>
<th>IUGR+V (n=8)</th>
<th>IUGR+SC (n=10)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Placental weight, g</td>
<td>559.7±35.0</td>
<td>376.2±32.5*</td>
<td>475.2±42.5</td>
<td>0.01</td>
</tr>
<tr>
<td>Fetal weight:placental weight</td>
<td>7.9 (7.1–8.3)</td>
<td>9.1 (6.7–10.1)</td>
<td>8.8 (7.9–9.8)</td>
<td>0.3</td>
</tr>
</tbody>
</table>
| Mean placenta
tome weight, g                 | 8.2±0.6             | 6.8±0.7           | 5.9±0.3†           | 0.02    |
| Placenta
tome number             | 70±5                | 57±4              | 80±6‡              | 0.01    |

Distribution of placentomes by morphological subtype

| % A                           | 33 (17–45)          | 41 (28–62)        | 26 (7–84)           | 0.53    |
| % B                           | 26 (20–39)          | 47 (31–58)        | 21 (15–43)          | 0.05    |
| % C                           | 19 (8–27)           | 10 (0–20)         | 31 (0–57)           | 0.36    |
| % D                           | 11 (1–34)           | 0* (0–0)          | 1 (0–9)             | 0.01    |

Distribution of placentomes by weight

| % small (<2 g)                | 7 (4–10)            | 10 (2–17)         | 16 (8–25)           | 0.16    |
| % medium (2–4 g)              | 15 (8–19)           | 15 (13–25)        | 23 (19–24)          | 0.10    |
| % large (>4 g)                | 79 (75–84)          | 70 (67–77)        | 63† (57–68)         | 0.02    |

Data are presented as mean±SEM or median (interquartile range) as appropriate; n=9, 8, and 10 (control, IUGR+V, and IUGR+SC). IUGR indicates intrauterine growth restriction; SC, sildenafil; and V, vehicle.

*P<0.05 vs controls (1-way ANOVA with Tukey multiple comparisons test or Kruskall–Wallis with Dunn multiple comparisons test as appropriate).
†P<0.05 vs IUGR+V (1-way ANOVA with Tukey multiple comparisons test).
function, blood gas and glucose concentrations were not available for every animal at every time point. Before the onset of embolization, there were no differences among groups in fetal arterial blood gas or glucose concentrations (Figure 2A through 2D). Embolization resulted in a tendency to increased fetal PaCO₂ and reduced fetal PaO₂ compared with controls; the differences in PaO₂ and PaCO₂ were statistically significant on days 105 and 106 (P<0.05 versus controls day 105; P<0.01 versus controls day 106; Figure 2B and 2C). During this period, fetal arterial glucose concentrations were also intermittently lower in the embolized group with significantly lower concentrations on days 105 (P<0.05) and 107 (P<0.01; Figure 2D). Fetal arterial pH was significantly lower in the embolized group on day 106 (P<0.05), but otherwise it was similar among groups (Figure 2A). There was no significant effect of sildenafil treatment on fetal arterial blood gas parameters or glucose concentrations (Figure 2).

Uterine Artery Blood Flow

Because of complications arising with venous catheter occlusions, only a small number of ewes completed the antipyrene protocol. Median or individual values of blood flow calculated for each of the time points are provided in the online-only Data Supplement.

Umbilical Artery Blood Flow

Mean umbilical artery RI did not differ among groups at any individual time point (Figure 3A); however, the mean change in RI for each fetus from 107 dGA (end of embolization) to 128 dGA (last measurement recorded) differed significantly among groups (F(2, 22)=4.21; P=0.03; Figure 3B). Over this time period, RI fell significantly less during the treatment period in the IUGR+V group compared with controls (mean change in RI[107–128 dGA] control versus IUGR+V versus IUGR+SC: –0.09±0.03 versus 0.01±0.02 versus –0.03±0.02; P=0.03). The change in RI for the lambs of sildenafil-treated mothers did not differ significantly from either of the other 2 groups.

Ex Vivo Vascular Function

There was no difference among groups in myometrial artery maximum constriction to phenylephrine, maximal relaxation to acetylcholine or sodium nitroprusside, or sensitivity to any of the 3 agents (as assessed by calculation of the EC50; Figure 4).

Discussion

Our study adds to the growing body of evidence that sildenafil can improve fetal growth and suggests that changes in growth are at least partly mediated by increased placental growth and reduced fetal–placental vascular resistance, as opposed to changes in maternal myometrial resistance vessel function.

Lamb weights of the IUGR+V group were significantly less than the control group, whereas lamb weight from the IUGR+SC group were not, suggesting that maternal sildenafil administration had a beneficial effect on fetal growth. Our study was powered to detect a relatively large (25%) difference in fetal weight between vehicle- and sildenafil-treated groups; however, the difference in lamb weights in the IUGR+V and IUGR+SC groups in our study was only 7%. Although this increase is modest, in the absence of any alternative therapies, even a treatment that produced small increases in fetal weight would be clinically useful. Clinically, the babies at the greatest risk of serious morbidity and mortality are those that are the most growth restricted.25 Perhaps most importantly, sildenafil reduced the proportion of fetuses with a weight less than the fifth centile of the control group, suggesting that treatment may have had a greater impact on the growth of the smallest fetuses.

Placental size is a determinant of adequate maternal–fetal exchange capacity, and small placentae are associated with

Figure 2. Fetal arterial pH (A), PaO₂ (B), PaCO₂ (C), and glucose concentrations (D) during maternal uterine artery embolization and treatment periods. Data are presented as median±interquartile range. For the embolization period, intrauterine growth restriction (IUGR)+vehicle (V) and IUGR+sildenafil (SC) are combined into a single IUGR group (n=15–18) vs control (n=5–6). For the treatment period, the IUGR groups are separated into vehicle treated (IUGR+V; n=4–8) vs sildenafil treated (IUGR+SC; n=4–9) vs control (n=1–6). *P<0.05 and †P<0.01 vs control (Mann–Whitney test); ‡P<0.05 for IUGR+V vs control (Kruskal–Wallis with Dunn multiple comparisons test); ●, control; ■, IUGR (IUGR+V for treatment period); and ▼, IUGR+SC.
both IUGR and adverse pregnancy outcomes.26,27 Placentae from IUGR pregnancies show pathological findings of acute atherosis, thrombosis, obliteration of the maternal artery lumen, and infarction.28,29 Ovine uterine artery embolization results in blockage of the small maternal arterioles supplying the placenta and results in placental infarction and thereby provides a paradigm with relevance to human pregnancies affected by IUGR.30 We observed a large drop in total placental weight in the IUGR+V group, whereas placental weight from the IUGR+SC group was intermediate to the other 2 groups, implying that sildenafil increased placental growth. The weight of fetal tissue produced per unit of placental weight was similar among groups, consistent with findings in small animal studies,15,17 suggesting that changes in fetal growth are because of an increase in placental mass rather than an increase in placental efficiency. We also observed a relative increase in the number of placentomes and an increase in proportion of smaller placentomes in the sildenafil-treated ewes. We speculate that this could represent sildenafil having a selective effect on growth at new or relatively underdeveloped sites of placentome attachment, as opposed to increased growth of all placentomes, including those that are already well established. From the findings of the present study alone, it is not possible to determine the mechanism through which sildenafil might increase placental (and therefore fetal) weight. However, there is some evidence from in vitro and small animal work that sildenafil has a proangiogenic function: endothelial cells show increased endothelial cell proliferation migration and organization after culture with sildenafil,31 and sildenafil promotes angiogenesis and increased blood flow in cardiomyocytes after cardiac ischemia reperfusion injury in rats.32 These changes were associated with an upregulation of the vascular endothelial growth factor system—a family of growth factors that play a critical role in all stages of placental development. It is feasible that an upregulation of the vascular endothelial growth factor system in the placenta could promote angiogenesis and result in the increase in placental mass observed in this study.

Our other finding of note is the change in umbilical artery RI; this suggest that maternal sildenafil administration reduced fetoplacental resistance. In human pregnancies affected by IUGR, a plateau or increase in the umbilical artery RI is a marker for fetal compromise and correlates with pathological placental findings, such as reduced volume of intermediate and terminal villi, reduced number, and diameter of fetal capillaries.33,34 In this study, the fall in RI was the greatest in the control group and least for the IUGR+V group, with IUGR+SC RI intermediate to the other groups, suggesting that sildenafil may help normalize placental resistance within the fetal–placental circulation. Similar observations have been made in murine studies, where pathological umbilical artery resistance indices were normalized when dams were treated with sildenafil during pregnancy.16 The mechanism behind a reduction in fetoplacental resistance is not clear but could arise from either an increased sensitivity of the fetoplacental vasculature to dilators or via a proangiogenic effect on the fetal vasculature, resulting in increased villous growth.
Contrary to our hypothesis, we did not observe an alteration in myometrial resistance vessel sensitivity to endothelial-dependent vasodilators. This is similar to observations in a small study of preeclamptic women but differs from that observed in mice. It is possible that sildenafil may have an effect on resistance vessel function that subsidises as tissue levels of sildenafil fall. The half-life of sildenafil is relatively short (~4 hours in humans); median collection time for arteries was >5 hours after last treatment administration in the human study and >24 hours in our study. This is in contrast to the murine study where treatment (administered in drinking water) was available up to the point of tissue collection.

Our results differ somewhat from the only other published report of chronic maternal sildenafil exposure in pregnant sheep. Satterfield et al used caloric restriction to induce growth restriction, treating pregnant ewes with sildenafil in thrice daily subcutaneous injections from 28 to 115 dGA. A 14% increase in fetal weight was seen with sildenafil treatment (150 mg per day), with no difference in placental weights between groups. The difference in findings between our studies may be consequent on the different methods of inducing growth restriction, durations of treatment (87 days in Satterfield et al versus 21 days in our study), or the different timings of treatment in relation to ovine placent al development. It is also possible that the slow subcutaneous infusion used in our experimental design did not result in sufficiently high peak plasma sildenafil concentrations for maximum effect. Irrespective of these differences, in the context of developing treatments for human pregnancy, it is worthwhile noting that neither study has shown a detrimental effect on fetal growth or fetal organ development. In particular, our study did not show a detrimental effect on fetal oxygenation. This is in contrast to the observed reduction in fetal oxygenation and mean arterial blood pressure accompanying a reduction in uterine blood flow seen in another study after a large intravenous bolus of sildenafil in the pregnant sheep.

Phosphodiesterase-5 is expressed in tissues other than the uterus, and the effect of sildenafil on other organ systems should also be considered. In women, reduced plasma volume increase is associated with IUGR. A selective increase in phosphodiesterase-5 activity has been demonstrated in the inner renal medulla of the pregnant rat, where it seems to play an important role in increasing sodium and water retention, resulting in increased plasma volume. In rodents, renal infusion of sildenafil has been associated with blunted antiatriuresis and increased diuresis, and oral administration has been associated with reduced maternal plasma volume. Changes in blood and plasma volumes are minimal in normal ovine gestation, so the changes described in rats may not be present in sheep. However, as we did not measure the ewe’s blood or plasma volume during this study, we are unable to clarify the effect of chronic sildenafil administration on maternal fluid homeostasis. Any future studies should consider incorporating measurements of blood or plasma volume to clarify this point.

**Perspectives**

This study suggests that maternal sildenafil treatment may be associated with a small increase in fetal weight and a modest increase in placental weight in fetal lambs where IUGR has been induced by uterine artery embolization. Treatment with sildenafil was not associated with changes in myometrial resistance artery function but was associated with a fall in umbilical artery resistance indices, suggesting increased fetal-placental perfusion. These findings, together with the absence of detrimental effect on organ growth, suggest that sildenafil may be a useful adjunct to the management of growth-restricted pregnancies.

**Acknowledgments**

We gratefully acknowledge the technical assistance of the Auckland University research farm technicians Travis Gunn, Maggie Worthington, Gregg Pardoe, and Anita Wylie.

**Sources of Funding**

This research was supported by GRA VIDA (National Centre for Growth and Development Scholarship) and funding from the Mercia Barnes Trust.

**Disclosures**

None.

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**Novelty and Significance**

**What Is New?**

- Rodent studies have suggested a positive effect of the phosphodiesterase-5 inhibitor sildenafil citrate on fetal growth, but the mechanism(s) of action remains unclear. Furthermore, the duration of treatment in these studies was considerably less than what would pertain in human pregnancies, so a continued beneficial effect and the absence of any detrimental effects on organ growth and development have not been proven. We have used a clinically relevant paradigm of intrauterine growth restriction to show a positive effect of sildenafil on fetal growth over a longer (and clinically appropriate) timeframe, and for the first time, we describe effects of medium-term sildenafil exposure on fetal–placental blood flow and myometrial resistance vessel function.

**What Is Relevant?**

- There are no clinically available treatments that can improve intrauterine growth. This study provides important evidence in support of the efficacy and safety of sildenafil as a new treatment for intrauterine growth restriction.

**Summary**

In pregnant ewes, uterine artery embolization resulted in reduced fetal and placental weights and an increase in fetal–placental vascular resistance (as assessed by Doppler ultrasound). These changes were all partially ameliorated when ewes were treated with sildenafil. Furthermore, no changes in resistance vessel function were observed, suggesting that changes in growth are at least partly mediated by increased placental growth and reduced fetal–placental vascular resistance, as opposed to changes in maternal myometrial resistance vessel function.
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Hypertension. 2016;68:760-767; originally published online July 18, 2016;
doi: 10.1161/HYPERTENSIONAHA.116.07662

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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Vascular Smooth Muscle Sirtuin-1 Protects Against Diet-Induced Aortic Stiffness

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Short Title: Smooth muscle sirtuin-1 prevents aortic stiffness

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MATERIALS AND METHODS

Experimental animal models. All animal experimental procedures were approved by the institutional animal care and use committee (IACUC) at Boston University Medical Campus. We generated SirT1 vascular smooth muscle knockout (SMKO) and transgenic (SMTG) mice on C57Bl/6J genetic background, in house, as described and characterized in 1. Specifically, mice with two loxP sites flanking SirT1 exon 4, required for the enzyme catalytic activity, were obtained from Dr. David Sinclair at Harvard University, Cambridge, MA, USA. Floxed SirT1<sup>ex4</sup> mice were bred with mice in which Cre-recombinase expression is driven by the endogenous smooth muscle 22 α protein (SM22α, transgelin) promoter (Jackson Laboratories; endogenous locus SM22α Cre Knock-In; B6.129S6 <i>Tgln<sup>tm2(cre)Yec</sup>/J</i>) to generate a mouse with SirT1 exon 4 deletion in smooth muscle (SMKO).

Similarly, mice with SirT1 gene inserted downstream of the collagen type I locus and preceded by loxP sites flanking a Stop codon (obtained from Dr. David Sinclair), were intercrossed with the SM22α-Cre males, described above, to generate mice over-expressing SirT1 in VSM (SMTG).

Mice over-expressing SirT1 globally (SirT1 Bacterial Artificial Chromosome Overexpressor, SirBACO)<sup>2</sup> were obtained from Dr. Wei Gu, Columbia University, New York, NY, and maintained on a C57Bl/6J genetic background. Mice were housed in rooms with 12-hour light/dark cycle and in groups of 3-4, with food and water <i>ad libitum</i>. In each experiment the appropriate littermate controls (floxed SirT1<sup>ex4</sup>/Cre- or floxed Stop-SirT1/Cre-) were used and indicated as WT.

Diet and drug treatments. At 8 weeks of age, SMTG, SMKO, SirBACO mice and littermate controls were fed normal or high fat, high sucrose diets for up to 8 months. Diets were as follows: normal diet, ND: 4.5 % fat, 0% sucrose; high fat, high sucrose diet, HFHS: 35.5 % fat from lard, representing 60 % calories, 16.4 % sucrose (catalog numbers D09071702 and D09071703, Research Diets, New Brunswick, NJ, USA). To note, the control diet (ND) was custom-formulated to match the micronutrients contained in HFHS, except for fat and sucrose.

Fasting was performed in subsets of WT or SMKO mice, fed ND or HFHS, for 2 or 8 months, by removing food overnight and measuring arterial stiffness, in the same mice, before (fed) and after food removal (fasted).

Subsets of male C57Bl/6J mice (stock number 00664, The Jackson Laboratory, Bar Harbor, ME, USA) were fed HFHS diet supplemented with resveratrol or S17834, two polyphenolic compounds known to activate SirT1<sup>3,4</sup>. Resveratrol and S17834 were dissolved in ethanol and admixed with food to obtain a daily dose of 130 mg/kg, allowing the ethanol to evaporate for 48 hrs before feeding the diet to mice.

In reversal studies, subsets of mice were fed HFHS for 8 months before receiving HFHS supplemented with SRT1720, a small molecule SirT1 activator, more specific than resveratrol<sup>3,5</sup>, for one week. SRT1720, was suspended in ethanol, sonicated until complete dissolution and mixed into HFHS diet, followed by ethanol evaporation, to achieve a dose of 100 mg/kg/day. Body weights were recorded at baseline and every month after diet initiation.

Glucose tolerance test (GTT). Glucose tolerance was assessed in HFHS-fed WT (n=5-9), SMTG (n=6), SirBACO (n=5) mice and mice treated with S17834 (n=9) after an overnight fast, as we previously described<sup>6</sup>. Briefly, plasma glucose levels were measured with a strip meter (Accu-Check, Roche Diagnostics, Indianapolis, IN, USA) before and every 30 min after intraperitoneal injection of a
freshly prepared glucose solution (1.5 mg/kg of body weight), over a 120-minute period. Data are expressed as curves of glucose plasma concentration (mg/dl) plotted against time (min) for each experimental group.

**Pulse wave velocity (PWV) measurements.** PWV is the gold standard *in vivo* measurement of arterial stiffness. PWV was assessed in the following experimental groups, where WT represents appropriate littermate controls, using methods that we previously described 1, 6, 7:

experiment 1) WT/ND before and after fasting (n=11), WT/HFHS before and after fasting (n=11) at 2 months of diet;
experiment 2) WT/ND before and after fasting (n=9), WT/HFHS before and after fasting (n=9), at 8 months of diet;
experiment 3) WT/HFHS before and after fasting (n=4), SMKO/HFHS before and after fasting (n=4) at 8 months of diet;
experiment 4) WT/HFHS (n=4), SMTG/HFHS (n=6) at baseline, 4 and 8 months of diet;
experiment 5) WT/ND (n=6), WT/HFHS (n=6), WT/HFHS/resveratrol (n=6) at baseline, 4 and 8 months of diet;
experiment 6) WT/ND (n=6), WT/HFHS (n=6), WT/HFHS/S17834 (n=6) at baseline, 4 and 8 months of diet;
experiment 7) WT/ND (n=5), WT/HFHS (n=5), SirBACO/ND (n=6), SirBACO/HFHS (n=7) at 8 months of diet;
experiment 8) WT/HFHS (n=4), WT/HFHS/SRT1720 (n=5) at 8 months of diet and after one week of drug treatment.

Briefly, mice were kept recumbent on a heated pad and attached to electrocardiogram (ECG) pads. A high-resolution Doppler echocardiography transducer (VEVO770, FujiFilm) was used to acquire 20 seconds of continuous recording of blood flow waveforms from 2 locations along the aorta, one proximal and one distal to the heart. The time between the R peak of ECG and the foot of each flow waveform were manually measured post-acquisition on at least 5 cardiac cycles, for the proximal and distal locations, for each mouse to compute the foot-to-foot arrival times. PWV (m/s) was calculated by dividing the distance between the proximal and distal locations by the difference in the proximal and distal arrival times. To note, PWV measurements were previously optimized by us 1, 6, 7 to maintain heart rates and mean arterial pressures at comparable values (heart rate 450 ± 30 bpm and mean arterial pressure 95 ± 5 mmHg) in all animals during Doppler waveform acquisitions.

**Aortic tissue isolation and ex vivo treatments.** Whole aortas were cleaned of periaortic fat and removed from PBS-perfused WT and SMTG mice. Aortas were cultured at 37°C overnight in 1 ml culture medium (DMEM containing 1g/mL glucose), with or without TNFα (10 ng/ml, Peprotech) or with or without SRT1720 (50 μmol/L, Sellekchem). At the end of the overnight incubation, aortas were briefly washed in cold PBS, snap-frozen in liquid nitrogen and kept at -80°C until further processing.

**Isolation of vascular smooth muscle cells.** Vascular smooth muscle (VSM) cells were isolated by enzymatic dissociation, as we previously described 1, 8. Aortas from WT and SMTG mice were cleaned of blood and periaortic fat and incubated at 37°C for 12 min in 3 mg/mL collagenase II (Worthington, Lakewood, NJ, USA), in serum-free DMEM. Adventitia was removed manually and the
medial layer was further dissociated in 3 mg/mL collagenase and 1 mg/mL elastase at 37°C for 30 min, with gentle agitation every 10 min. Cells were centrifuged and resuspended in complete growth medium (DMEM, 1 g/L glucose supplemented with 10% fetal bovine serum and 1% antibiotic-antimycotic) and used until passage 5.

**Western blot on aortas and VSM cells.** Aortas and VSM cells were manually homogenized in a glass-glass grinder (Kontes, number 20) in RIPA buffer (200 µL/1 mg tissue, Cell Signaling Technology, Danvers, MA, USA), freshly supplemented with protease inhibitor cocktail and trichostatin A (5 µM). Homogenates were sonicated (10 seconds, x3, on ice) then cleared of non-soluble material by centrifugation at 14,000 rpm for 10 min, 4°C. Protein concentration was assessed using Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL, USA), as per manufacturer’s instructions.

VSM cell or aortic lysates were resolved by SDS-PAGE in 4-20% or 7.5% polyacrylamide gels and transferred to PVDF membranes (100V, 120 min). Membranes were blocked in 5% non-fat milk in 1% Tween-20 in TBS (TBS-T) for 1 hour and incubated with one of following antibodies, overnight at 1:1000 dilution: SirT1 (catalog # ab110304, Abcam, Cambridge, MA, USA), VCAM-1 (catalog# 14694, Cell Signaling Technologies), phosphorylated p-65 NFκB (catalog# 3033S, Cell Signaling Technologies), acetylated histone H3 (catalog# 9649S, Cell Signaling Technologies), p47phox (catalog# BS4600, Bioworld Technologies), phosphorylated VASP (catalog# 3114, Cell Signaling Technologies), β-actin (catalog# A5441, Sigma-Aldrich) and GAPDH (catalog # 2118S, Cell Signaling Technologies). Membranes were then washed in TBS-T and incubated with peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Cell Signaling Technologies) for 1 hour then washed in TBS-T and exposed to the chemiluminescent substrate ECL (GE Healthcare, Boston, MA, USA) to visualize protein bands. Band images were documented with Imager model # LAS4000 (General Electric). Protein band intensities were quantified with Image J (www.nih.gov), with band intensities pertaining to the same experimental group averaged and normalized to β-actin or GAPDH, used as loading controls, and expressed as fold change relative to WT controls.

**SirT1 mRNA quantitation by qRT-PCR.** Aorta, isolated from WT (n=4) and SMTG (n=4) mice, were homogenized in Trizol and kept at -80°C until RNA extraction by chloroform separation/isopropanol precipitation. Messenger RNA was retrotranscribed into cDNA with High capacity RNA-to-DNA kit (Life Technologies), as per manufacturer’s instructions. SirT1 gene expression was analyzed by quantitative RT-PCR with validated Taqman assays multiplexed for β-actin, in AB750 instrument (Applied Biosystems). SirT1 mRNA expression was calculated by the ΔΔCt method, using β-actin as endogenous control and expressed as fold ratio versus WT controls.

**NFκB activity assay.** WT and SMTG VSM cells, isolated as described above, were plated at a density of 5,000 cells/well in 96-well plates, in triplicate for each treatment. Cells were infected with adenoviral construct containing a firefly luciferase reporter gene under the control of specific NFκB DNA responsive elements 9 (1 x 10⁹ PFU/ml) and a renilla luciferase (1 x 10⁹ PFU/ml), as internal control, in DMEM medium without serum or antibiotics. Four hours post-infection, complete growing medium (DMEM with 10% FBS and 1% antibiotics) was added. Two days later, cells were starved overnight in minimal medium (DMEM with 0.5% FBS) then treated overnight with TNFα (10 ng/ml)
or vehicle control (PBS). Cells were then lysed and the firefly and renilla luciferase enzymatic activities were assayed with a standard luciferase assay, as per manufacturer’s instructions (Promega). The experiment was repeated four times.

**Oxidant measurement in the aorta.** Oxidants were assessed in fresh frozen aortic sections with dihydroethidine (DHE), as we previously described \(^1\). Briefly, ~ 1 cm of thoracic aorta from ND- and HFHS-fed WT, SirBACO and SRT1720-treated mice (n=5, WT/ND; n=10, WT/HFHS; n=5, SirBACO/HFHS and n=4, WT/HFHS+SRT1720) were frozen in OCT and kept at -80°C until sectioning with a cryotome at a thickness of 10 µm. Aortic sections were washed on ice with cold PBS and incubated with DHE solution, freshly prepared at 10 µM working solution, at 37°C for 45 min. Sections were then washed in PBS and cover-slipped with Prolong Gold Antifade mounting medium (Life Technologies, Grand Island, NY). Digital images were captured with an epifluorescent microscope (Nikon Eclipse 80i), a CDD camera (DQ Qi Mc), and NIS-Elements 3.22 software (Nikon, Melville, NY, USA). DHE red fluorescence intensity was quantified by 6 independent observers, blinded to the experimental groups with a scoring system on a scale 0-4, with 0 corresponding to the lowest intensity and 4 to the highest. Scores pertaining to the same experimental group were averaged before statistical analysis.

**Immunohistochemistry on aortic sections.** We evaluated the expression of NAPDH oxidase Nox2 in freshly frozen aortic sections prepared from HFHS-fed WT (n=5) and HFHS-fed SMTG (n=6), by standard immunohistochemical methods using Vactastain ABC kit (Vector), as we previously described \(^2\).

**Statistical analysis.** All data are expressed as mean ± SEM and analyzed with GraphPad Prism v.5.0c. Repeated measures two-way ANOVA with Bonferroni’s multiple comparison post-hoc test was used to analyze PWV and body weights in SMTG (Figure 2A and S2A) and in mice treated or untreated with resveratrol or S17834 (Figures 2B, S2C and S2D), during the time course of ND or HFHS diets, and to analyze GTT in HFHS-fed WT, SMTG, SirBACO and S17834-treated mice (Figures S2B, S2F and S2G). Body weights in SirBACO mice (Figure 2E), PWV values in fasting experiments (Figure 1A), logarithmically transformed to fit a normal distribution, PWV values in fasted SMKO mice (Figure 1B) and NFκB activity luciferase assay (Figure 3C) were analyzed by one-way ANOVA with Bonferroni’s multiple comparison post test. PWV in SirBACO mice (Figure 2C) and quantification of DHE staining (Figure 4A) were analyzed by nonparametric one-way ANOVA Kruskal-Wallis test with Dunn’s multiple comparisons post test. PWV and body weights in mice treated with or without SRT1720 (Figure 2D) were analyzed by unpaired t-test. When experiments had n < 7, such as Western blots and qRT-PCR, non parametric Whitney-Mann t test was used to compare the means of two groups. P values < 0.05 were considered significant.
AUTHORS CONTRIBUTIONS.

JLF contributed to design the study and conducted experiments; LAS genotyped mice and performed Western blots and Western blots quantitations; RW contributed to design the study and performed PWV measurements; IVR performed Western blot, Western blot quantitations, and DHE immunostainings; XW supervised mouse feeding and recorded body weights over time; RAC and MB provided critical comments to the study and the manuscript; FS contributed to the study design, coordinated the study, designed and performed experiments, analyzed the data and wrote the manuscript.
REFERENCES FOR ONLINE SUPPLEMENTS.


Figure S1. Characterization of SMTG mice. (A) Representative Western blots of aortas (with adventitia removed; right panel) and VSMC isolated from aortas (left panel) of WT and SMTG mice, confirming SirT1 over-expression in VSM. Each lane represents one mouse. (B) Quantitative RT-PCR for SirT1 indicates ~4-fold increase in SMTG aortas (n=4) compared with WT (n=4). *, p<0.05.
Figure S2

Figure S2. SirT1 activation does not have metabolic effects in HFHS-fed mice. (A) Body weights in WT (n=4) and SMTG (n=6) fed HFHS up to 8 months. (B) Glucose tolerance test in HFHS-fed WT (n=4) and SMTG (n=6) mice, performed as described in methods. Curves plot glucose plasma concentration over time, for the two groups. Body weight gains over 8 months in mice fed (C) ND (n=6), HFHS (n=6) or HFHS supplemented with resveratrol (130 mg/kg/day; n=6) or (D) ND (n=6), HFHS (n=6) or HFHS supplemented with S17834 (130 mg/kg/day; n=6). *, p<0.05 WT/HFHS vs WT/ND. †, p<0.05 WT/HFHS/resv or WT/HFHS/S17834 vs WT/ND. (E) Body weights were similarly increased in HFHS-fed SirBACO mice as in HFHS-fed WT littermate controls, after 8 months of HFHS. *, p<0.05 WT/HFHS (n=5) or SirBACO/HFHS (n=6) vs WT/ND (n=5). (F-G) Glucose tolerance test in HFHS-fed WT (n=5-9), SirBACO (n=5) and S17384-treated (n=9) mice, performed as described in methods.
Figure S3.

The polyphenol S17834 protects against HFHS-induced arterial stiffness. The polyphenolic compound S17834, administered in food for 8 months at a dose of 130 mg/kg/day, prevented HFHS-induced arterial stiffness, measured by PWV (m/s). ND, normal diet. *, p<0.05 ND (n=6) vs HFHS (n=6); †, p<0.05 HFHS/S17834 (n=6) vs HFHS (n=6).
Figure S4

Figure S4. Nox2 expression in the aorta of HFHS-fed mice. Representative images, at 40x magnification, of aortic sections from HFHS-fed WT (n=5) and SMTG (n=6) mice immuno-stained with an antibody against Nox2. An aortic section processed in absence of the primary antibody served as a negative control, to prove the specificity of the signal. E, endothelium; M, media; A, adventitia.