Antenatal sildenafil citrate treatment increases offspring blood pressure in the placental-specific \( \text{Igf2} \) knockout mouse model of FGR

© L. J. Renshall, E. C. Cottrell, E. Cowley, C. P. Sibley, P. N. Baker, E. B. Thorstensen, S. L. Greenwood, M. Wareing, and M. R. Dilworth.

Antenatal sildenafil citrate treatment increases offspring blood pressure in the placental-specific \( \text{Igf2} \) knockout mouse model of FGR. *Am J Physiol Heart Circ Physiol* 318: H252–H263, 2020. First published December 6, 2019; doi:10.1152/ajpheart.00568.2019.

**INTRODUCTION**

Fetal growth restriction (FGR), the inability of a fetus to reach its predetermined genetic growth potential, is a major complication of pregnancy affecting up to 8% of births in the UK (13). FGR infants are at increased risk of mortality and morbidity both in the short and longer term (2, 25). FGR fetuses are 10 times more likely to be stillborn than appropriately grown fetuses (1, 4, 15). There are no current therapies for FGR, in part due to the possible teratogenic effects of pharmaceutical agents (48). This risk has led to a reluctance within the pharmaceutical industry to develop drugs for obstetric complications and the repurposing of therapies currently used in nonpregnant individuals (8, 14, 39). In the absence of treatments, in cases of severe early onset FGR, the only clinical option is premature delivery of the baby, which, in itself, increases the risk of adverse effects on maternal and fetal health (22) and is associated with increased morbidity in adulthood (9, 21).

One drug that has been repurposed as a potential therapeutic for FGR is the potent vasodilator sildenafil citrate (SC). SC (marketed as Viagra) is a selective phosphodiesterase type 5 (PDE-5) inhibitor that inhibits the hydrolysis of cyclic guanosine monophosphate (cGMP), thus prolonging the actions of the vasodilator nitric oxide (NO). SC dilated myometrial arteries of women with FGR ex vivo but was without effect on arteries from normal pregnancies (47). In preclinical studies, SC treatment increased fetal weight in the catechol-\( O \)-methyltransferase knockout (\( \text{COMT}^{\text{–/–}} \)) mouse model of FGR by improving aberrant umbilical artery blood flow (43). We (10) also demonstrated that SC, administered via drinking water, increased the weight of FGR fetuses of the placental-specific insulin-like growth factor 2 (\( \text{Igf2} \) \( \text{PO}^{\text{+/+}} \)) knockout mouse via an overall increase in nutrient transfer capacity of the placenta. In two separate studies, SC treatment in growth-restricted ovine fetuses also led to an increase in fetal weight as a result of an increase in nutrient transfer capacity of the placenta (30, 38). However, in the single umbilical artery ligation (SUAl) sheep model of FGR, SC led to reduced uterine blood flow as well as reduced \( \text{PO}_2 \), hypotension, and tachycardia in fetuses...
from both normal and SUAL ewes (27). Overall, these data suggest that the underlying etiology of FGR may determine whether SC is beneficial.

Following these preclinical studies, and a small nonrandomized clinical trial suggesting that maternal SC may increase fetal abdominal growth velocity (45), the multicenter randomized control trial “Sildenafil Therapy In Dismal Prognosis Severe Early Onset IUGR” (STRIDER) commenced. Despite the wealth of preclinical data suggesting effectiveness of SC at increasing fetal growth, the clinical trial found that SC, compared with placebo, did not prolong pregnancy, or have any effect on fetal growth velocity or fetal or neonatal survival rates (18, 41). Furthermore, the Dutch STRIDER trial was halted, as there was an increased incidence of lung complications in babies from mothers who had taken SC during pregnancy (19). The question of whether antenatal treatment with SC resulted in long-term health implications for the offspring remains unanswered following these trials. However, recent preclinical data demonstrated that treating endothelial nitric oxide synthase knockout (eNOS<sup>−/−</sup>) mice with SC during pregnancy resulted in a significant rise in systolic blood pressure (SBP), which was associated with a constrictive phenotype in mesenteric arteries (28). In the same study, C57BL/6J offspring from dams exposed to SC during pregnancy showed increased endothelial-dependent relaxation in isolated mesenteric arteries with no change in SBP; these data suggest that eNOS<sup>−/−</sup> mice may be more vulnerable to negative effects associated with SC. We therefore chose to assess the long-term implications of antenatal SC in the Igf2 P0<sup>−/−</sup> (P0) knockout mouse, as this model of FGR is not characterized by a cardiovascular phenotype but does show evidence of altered placental morphology and function akin to human FGR (7, 12, 42).

For this study, we sought to reproduce the concentration of SC in maternal blood from previous (35) and recently completed clinical trials (18, 41). Pregnant dams were therefore given a subcutaneous injection of 10 mg/kg SC or saline. Postnatal weight gain, glucose tolerance, blood pressure, and resistance artery function in adult male and female offspring of both wild-type (WT) and P0 genotypes were then assessed. We hypothesized that maternal SC treatment of the P0 knockout mouse would have no detrimental effects on cardiovascular function of the offspring.

METHODS

**Ethical Approval**

All procedures were performed in accordance with the UK Animals Scientific Procedures Act (1986) and under the provision of a UK Home Office project license (PPL 40/3385 and P9735892D). Work was approved by the local animal welfare and ethical review board of the University of Manchester. This study is reported according to the ARRIVE guidelines (23). Mice were fed a standard pellet diet (BK001 diet, Special Dietary Services) with ad libitum access to water (Hydropac, Laboratory Products) and were caged in individually ventilated cages under a 12-h:12-h light-dark cycle at 21–23°C with 65% humidity.

**P0<sup>−/−</sup> Mouse**

Males (12–26 wk old) heterozygous for the deletion of the P0 transcript were mated with 8- to 12-wk-old virgin C57BL/6J (WT) females. A total of 76 females were mated, with 48 confirmed pregnancies. Identification of a vaginal plug the following morning was deemed to be the beginning of pregnancy and designated embryonic day 0.5 (E0.5; term ~E19.5). Litters contained both WT and P0 (growth-restricted) fetuses. Mice were originally a kind gift from Wolf Reik and Miguel Constância (6).

**SC Treatment**

To assess the effects of antenatal SC treatment on fetal and placental weight, litter size, and viability, dams (the experimental unit) were randomly assigned to receive either a subcutaneous injection in the skinfold between the scapulae of 10 mg/kg sc (Pfizer, UK, n = 10 dams) or 0.9% saline (n = 12 dams; control) daily (9:00–10:00 AM) from E12.5 up to and including E17.5 (see Fig. 1 for study design). Maternal body weight at E18.5 was similar between saline and SC groups (38.8 ± 1.4 and 38.4 ± 1.0 g, respectively; means ± SE). Saline and SC treatment regimens were performed in parallel. We determined the animal equivalent dose [AED (29)] in mouse based on the amount of SC received by women recruited into the STRIDER clinical trial (25 mg 3 times/day, i.e., a total of 75 mg/day). The AED approximated to 13.2 mg/kg based on a pregnant woman with a weight of 70 kg, but this was reduced to 10 mg/kg in mouse to compare with similar studies in rat (36). We employed a daily subcutaneous injection dosing regimen, thus ensuring that mice received an exact dose of SC as opposed to previous treatments in drinking water (10, 28, 43). A separate set of experiments were performed on offspring (the experimental unit) from dams that were administered either SC (n = 11 dams) or saline (n = 9 dams) as above.

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** Study design. Number of litters and pups [wild type (WT) and placental-specific insulin-like growth factor 2 knockout (P0)] in each of the treatment groups are shown. For each treatment group, litters were separated into 2 study arms. The fetal study arm assessed effects of maternal sildenafil citrate (SC) treatment on fetal weight at embryonic day (E)18.5, whereas the offspring study arm assessed effects of antenatal SC treatment on postnatal body weight, glucose tolerance, systolic blood pressure (SBP), resistance artery function, and organ allometry. Terminal blood samples, used for measuring SC and desmethylsildenafil (d-SC), were taken between E17.5 and E18.5 from dams/fetuses treated with SC.
but then were allowed to litter down. We chose to assess outcomes from all pups from each of the litters for two reasons: 1) we were unable to reliably identify WT and P0 genotype until ear clipping at 4 wk of age; and 2) there were consistently lower survival rates of P0 offspring (between birth and 4 wk of age); thus, to increase the likelihood of at least one P0 offspring being present within a litter, all male and female offspring were used in all experiments.

**Measurement of Maternal and Fetal Plasma SC Concentration**

In a separate set of experiments 10- to 13-wk-old dams (n = 3) were euthanized by cervical dislocation 1 h (n = 1 dam) or 24 h (n = 2 dams) after a single injection of 10 mg/kg SC at E17.5. Following decapitation, trunk blood samples were collected in lithium-heparinized capillary tubes (Sarstedt). Blood from all fetuses (WT and P0 genotypes and both sexes) from each of the dams was also pooled for fetal measurements (1-h time point, 1 litter; 24-h time point, 2 litters). Blood was centrifuged at 1,900 g for 5 min, and plasma was collected and stored at −80°C. SC and its breakdown metabolite desmethyl-sildenafil (d-SC) were measured using triple-quadrupole mass spectrometry utilizing previously described methods (49), with minor modifications. Briefly 20 μL of internal standard solution (200 ng/mL SC-d3 in water) and 20 μL of 0.02 M aqueous NaOH was added to 100 μL of plasma and mixed. The analytes were extracted using 1 mL of ethyl acetate (Merck, Darmstadt, Germany). After removal of the organic supernatant to a clean tube, samples were dried by vacuum at E18.5 and offspring at the age of EC80 precontraction to U46619. Offspring Organ Allometry

**Postnatal Body Weight**

We attempted to standardize litters and weigh pups from birth. However, there were higher rates of mortality in P0 male and P0 female neonates than in WT fetuses, which was associated with maternal cannibalism. To avoid bias introduced by maternal interference, we chose to breed a minimum of nine litters for offspring studies. Offspring were caged with dams until weaning at 4 wk of age. At 4 wk of age, offspring were ear notched to aid identification of individual mice. A maximum of four female mice were housed in one cage. Male mice were separated and singly housed from 8 wk of age, as males were found to be aggressive toward littermates following separation for blood pressure measurements. Body weights were measured each week between weeks 5 and 12. Terminal body weights at week 14 were also measured. The mean body weight at each age was calculated for individual groups.

**Glucose Tolerance Test**

At 12 wk of age, mice were fasted overnight for 16 h with ad libitum access to water. A venipuncture was made at the base of the tail with a 25-gauge needle, and baseline blood glucose concentrations were measured using Accu-Chek Aviva (Roche Diagnostics) blood glucose monitoring system. Mice were then administered a 1 g/kg glucose (0.9% NaCl, 10% glucose) solution via intraperitoneal injection. Blood glucose concentrations at 15, 30, 60, 90, and 120 min postinjection were recorded.

**SBP Measurements in Offspring**

SBP was measured at 8 and 13 wk of age (see Fig. 1 for study design) using a noninvasive blood pressure system (model LE5001, Panlab). Mice were acclimatized to the restraint tube to reduce stress before SBP measurements. Mice were transferred to a room with a temperature between 22 and 24°C on the day of measurement and left to acclimate for 30 min before measurements were recorded. First, a small rodent restraining tube was placed on a Thermopad heated mat (36°C, Harvard Apparatus); mice were not forced to enter the restraint tube, but when they entered they were secured with the tail exposed for SBP measurements. The tail cuff occlusion device and pulse transducer were placed on the tail for at least 10 min before recording of SBP. Heart rate was measured and did not exceed 700 beats/min. A total of 12 SBP measurements were recorded within 30 min and averaged (mean) for each individual mouse.

**Mesenteric Artery Function in Offspring**

Second-order mesenteric arteries were harvested from offspring (14 wk) and mounted on a wire myograph (Danish Myo Technologies). Arteries were equilibrated and gassed with 20% O2-5% CO2, balance N2 and normalized to 0.9 of luminal pressure (L)13.3 kPa. Mesenteric artery contraction was assessed in response to depolarizing KPSS solution and U46619, (thromboxane A2 mimetic; 0.1–2,000 nM). Cumulative dose response curves were constructed and used to determine EC50 values. Mesenteric arteries were then precontracted with an EC50 concentration of U46619, before endothelium-dependent [acetylcholine (ACh); 0.1–10,000 nM] and independent [sodium nitroprusside (SNP); 0.1–10,000 nM] relaxation was measured. Contraction was expressed as active effective pressure using the following equation: active effective pressure = (wall tension/2π)/vessel internal circumference. Relaxation was expressed as a percentage of EC50 precontraction to U46619.

**Offspring Organ Allometry**

At 14 wk of age heart, kidney, spleen, lung, and brain were dissected from each mouse and weighed and presented as a percentage of body weight.
**Statistical Analyses**

Studies were primarily powered to detect changes in fetal weight and offspring blood pressure. All studies were based on 80% statistical power at a 5% significance level.

**Fetal and placental weight.** Data are presented as litter average (means ± SE) fetal or placental weight. Data were analyzed using two-way ANOVA with Bonferroni post hoc test.

**Body weight.** The level of significant difference was determined using mean body weight for each group with repeated-measures two-way ANOVA and Sidak's post hoc test.

**Glucose tolerance.** Total area under the curve (AUC) values were calculated for each individual mouse. Mean AUC values for each group were calculated and used for statistical analyses. A Mann-Whitney U-test was used to analyze mean AUC for non-normally distributed data.

**Blood pressure and mesenteric artery function.** Data are presented as means ± SE. Data were analyzed using two-way ANOVA with Bonferroni post hoc test.

**Offspring organ allometry.** Data are presented as median [min–max]. To assess the effect of genotype and sex within a particular treatment group (i.e., saline or SC) a Kruskal-Wallis test was used. A Mann-Whitney U-test was used to compare the effect of SC treatment between two groups; e.g., WT female (F) from SC-treated pregnancy vs. WT F from saline-treated pregnancy.

**RESULTS**

**Maternal and Fetal Plasma Measurements of SC and d-SC**

In maternal plasma, SC and d-SC reached a peak of 693 and 27 ng/mL, respectively, 1 h postinjection (n = 1 dam) and declined to 26 [2–51] (SC) and 2 [1–3] (d-SC) ng/mL after 24 h (median [min–max], n = 2 dams). SC and d-SC reached 42 [26–59] and 6 [5–7] ng/mL in fetal plasma 1 h after injection (n = 1 litter) and SC and d-SC declined to 2 [2–2] and 1 [1–1] ng/mL after 24 h (n = 2 litters), respectively.

**Litter Characteristics**

The mean number of fetuses per litter was not significantly different between saline control and treated groups (8.3 ± 0.3 vs. 7.8 ± 0.5, respectively, P = 0.48). SC had no effect on the mean number of fetal resorptions per litter compared with saline control (0.4 ± 0.1 vs. 0.8 ± 0.3, respectively, P = 0.20). SC did not affect the WT/P0 ratio of fetuses in each litter. In accord with previous studies (7, 10, 24, 34), fetal weight was significantly reduced in P0 vs. WT mice (P = 0.0001; Fig. 2A). SC had no effect on WT or P0 fetal weight at E18.5 (Fig. 2A).

A frequency distribution curve of fetal weights from saline control and SC-treated dams is shown in Fig. 2B. The 5th centile of saline control WT fetal weights was 1.08 g, with 70% of P0 littermates falling below this 5th centile value; 70% of P0 mice from SC-treated dams also fell below the 5th centile of saline control WT weight.

Consistent with previous findings, placental weight was significantly reduced in P0 mice vs. WT littermates (P < 0.001; Fig. 2C). SC did not affect placental weight in either WT or P0 mice (Fig. 2C). There was a significant increase in fetal-to-placental weight ratio in P0 fetuses compared with WT, independent of treatment (P < 0.01; Fig. 2D). However,
post hoc analysis showed no further differences in fetal/placental weight ratio between individual groups.

**Postnatal Growth**

Body weights from offspring of SC-treated pregnancies were compared with offspring from saline-treated pregnancies (Fig. 3). SC treatment did not affect body weight of male offspring between 5 and 12 wk of age irrespective of genotype (Fig. 3, A and C). Female P0 offspring exposed to SC in utero were similar in body weight to female offspring from saline-treated pregnancies (Fig. 3D). However, WT female offspring from SC-treated pregnancies were significantly heavier than WT female offspring from saline-treated pregnancies (Fig. 3B, \( P < 0.05 \)); this difference was significant at postnatal week 5 (17.3 ± 0.3 vs. 15.8 ± 0.4 g, respectively) and week 6 (19.0 ± 0.3 vs. 17.3 ± 0.4 g, respectively). There were no differences in body weight when glucose tolerance and SBP were first measured.

**Postnatal Glucose Tolerance**

Antenatal SC treatment had no effect on blood glucose concentration either before injection (fasting) or at any time point post-glucose challenge in male offspring compared with offspring of saline-treated dams irrespective of genotype (Fig. 4, A and C). Conversely, antenatal SC treatment significantly increased blood glucose concentrations in female offspring from both WT (saline AUC: 1,036 ± 28.6, SC AUC: 1,142 ± 24.5, means ± SE) and P0 (saline AUC: 940 ± 36.9, SC AUC: 1,123 ± 55.5) genotypes (\( P < 0.01 \) and \( P < 0.05 \), respectively; Fig. 4, B and D) compared with control offspring from saline-treated dams. In WT female offspring, this increase was most significant at \( t = 30 \) min (saline: 10.2 ± 0.4, SC: 11.9 ± 0.5 mmol/L, \( P < 0.01 \)) and \( t = 60 \) min (saline: 8.0 ± 0.4, SC: 9.1 ± 0.3, \( P < 0.05 \)), but there was no difference in fasting blood glucose concentration (Fig. 4B). In P0 female offspring, this increase was most significant at \( t = 30 \) min (saline: 9.2 ± 0.6, SC: 12.5 ± 0.6, \( P < 0.01 \)) and \( t = 90 \) min (saline: 6.3 ± 0.4, SC: 7.6 ± 0.4, \( P < 0.05 \)), but again there were no differences in fasting blood glucose concentration (\( t = 0 \) min; Fig. 4D).

**Postnatal SBP**

At 8 and 13 wk of age, SBP was not different when the effect of genotype on offspring from saline-treated pregnancies was compared (Fig. 5).

Independently of sex or genotype, maternal administration of SC led to a significant increase in SBP in 8- and 13-wk-old offspring compared with offspring from saline-treated pregnancies (\( P < 0.01 \)− \( P < 0.0001 \); Fig. 5, A−D).

**Postnatal Mesenteric Artery Vascular Function**

There was a significant reduction in U46619-induced contraction of WT male offspring mesenteric arteries from SC-treated pregnancies compared with WT male offspring from
saline-treated dams (Fig. 6A, P < 0.01), but this effect was not observed in P0 male offspring (Fig. 6B). There was an increased ACh-induced relaxation of WT male mesenteric arteries in offspring from SC-treated dams (Fig. 6C, P < 0.05) compared with saline-treated dams. Conversely, SC led to a reduced ACh-induced relaxation of mesenteric arteries from P0 males compared with P0 offspring from saline-treated pregnancies (P < 0.05; Fig. 6D). SNP-induced relaxation of mesenteric arteries from WT male offspring was reduced from SC-treated pregnancies compared with saline controls (P < 0.01; Table 1). Conversely, heart weight of P0 male offspring was increased by antenatal SC treatment vs. saline treatment (P < 0.05; Table 1).

DISCUSSION

There are currently no treatments for FGR. SC has been considered a promising therapeutic because of its beneficial effects on fetal growth in a number of animal models of FGR. However, recent randomized clinical trials have demonstrated no effect of SC on fetal growth in cases of severe early onset FGR (18, 41); effects on the offspring of these pregnancies remain unknown. In this study, we have demonstrated, contrary to our hypothesis, that exposure to SC increases SBP and impairs glucose tolerance in adult mouse offspring. Additionally, we have shown that subcutaneous administration of SC has no effect on fetal growth.

Litter Characteristics

In the COMT−/− and P0 mouse models of FGR, SC treatment, delivered via drinking water, led to an increase in fetal weight (10, 43) and, in the COMT−/− mouse, normalized abnormal umbilical artery Doppler waveforms (43). In the present study, we observed a growth-restricted phenotype consistent with our previous reports on the P0 mouse (7, 10, 12, 43).
Also in keeping with our previous reports (10), there were no effects of SC on litter size or number of resorptions in P0 mice. However, in contrast to the study of Dilworth et al. (10), where SC was given via drinking water, subcutaneous administration of SC to P0 dams at 10 mg/kg daily (E12.5–E17.5) in the present study did not increase fetal or placental weight.

The disparity between results from within our group in the effectiveness of SC to increase fetal weight in the P0 mouse would seem to be a consequence of dosing regimen. Unlike in our earlier study (10), we chose to use a subcutaneous injection of SC as a means to standardize the therapeutic concentration of SC across dams. Furthermore, our dosing regimen equates to a human equivalent dose (29) of 56.7 mg SC per day (based on a pregnancy weight of 70 kg), which is within the range of, and no higher than, dosages used in human clinical trials (16, 18, 35, 41). By use of this approach, maternal plasma showed a peak SC and d-SC concentration (693 and 27 ng/mL, respectively) 1 h postinjection. Both SC and d-SC were still present 24 h postinjection but had reduced significantly. Fetal plasma showed a peak SC and d-SC concentration (27 and 6 ng/mL, respectively) 1 h postinjection, and both were still measurable at 24 h postinjection but had declined significantly. Importantly, maternal and fetal SC concentrations were well within maximum tolerated doses (46). Both SC and d-SC have been detected in rat fetal liver after maternal oral administration of SC (31), but this is the first study to directly measure SC and d-SC in mouse fetal plasma and as such the first to confirm SC transfer across the mouse placenta. Previous preclinical studies showing beneficial effects of SC on fetal weight had given mice ad libitum access to SC in water, whereas our study involved a single daily dose of SC via subcutaneous injection. The doses used in previous studies equate to a human equivalent dose of ~228 mg (43) and 456 mg (10) of SC per day and are therefore higher than the dosage used in our study. Such differences in dosing regimen may underlie the differential efficacy of SC to increase fetal growth.

**Postnatal Weight**

Although it was not the primary focus of our study, we demonstrated that P0 mice, irrespective of sex, have similar growth trajectories from postnatal week 5 compared with WT littersmates. A recent publication by Mikaelsson et al. (26) demonstrated that P0 mice weighed 25% less than WT littermates at birth but demonstrated accelerated growth between 25 and 50 days of age, resulting in P0 mice having a near-normal, but still 7% lighter, body weight at postnatal day 100. However, Mikaelsson et al. did not assess the effect of sex on postnatal growth; thus, comparisons between the studies can be made only among male mice. It is also important to note that Mikaelsson et al. utilized the CD1 genetic strain of mouse.
which have a larger litter size resulting in reduction in birth weight and increased competition during the lactation period compared with the C57BL/6J background strain used in this study.

Male mice exposed to SC in utero had similar body weights to their equivalent WT or P0 saline controls. However, WT female offspring exposed to SC in utero had significantly increased body weights at 5 and 6 wk of age compared with saline controls; this effect was not observed in P0 female offspring. The mechanisms for increased body weight in WT female mice from SC-treated dams are unknown.

Glucose Tolerance

Blood glucose concentration was significantly higher in both WT and P0 female offspring from SC-treated dams compared with corresponding saline controls. Unlike studies purporting to show that females are protected against metabolic syndrome in cases of high-fat diet (32), treatment of dams with SC selectively reduced glucose sensitivity in female but not male offspring. Combined with the effect of antenatal SC on female offspring growth, these data suggest that female mice are more susceptible to the effects of antenatal SC. A recent study suggests that

Fig. 6. Dose-response curves of mesenteric arteries of male offspring from saline-treated and sildenafil citrate (SC)-treated pregnancies. A and B: mesenteric artery contraction in response to increasing doses of U46619 in wild-type (WT; left) and placental-specific insulin-like growth factor 2 knockout (P0; right) mice. C and D: relaxation of mesenteric arteries in response to ACh. E and F: relaxation of mesenteric arteries in response to SNP. Data are presented as means ± SE; number of offspring per group in parenthesis. Data were compared using 2-way ANOVA for effect of antenatal treatment with Bonferroni post hoc test. ACh, acetylcholine; SNP, sodium nitroprusside. For post hoc test, **P < 0.01.
exposure to SC in utero potentiates the impaired glucose tolerance and increases insulin resistance in male C57BL/6J offspring at postnatal day 150, when they are exposed to a secondary insult such as a high-fat diet (28). Our study and that of Mills et al. suggest that SC in utero may predispose offspring from normal pregnancies to glucose intolerance in later life.

Systolic Blood Pressure

FGR babies are at increased risk of hypertension in later life, which is often associated with metabolic syndrome (20). We are the first, to the best of our knowledge, to assess SBP in both male and female offspring of the P0 mouse. Previous studies have shown that male P0 offspring are growth restricted at birth, demonstrate early life catch-up growth, but continue to be 7% lighter than WT littermates by 100 days of age (26). Although P0 fetuses are growth restricted near term, we saw no differences in SBP at 8 or 13 wk of age when we compared P0 and WT offspring from saline-treated pregnancies. A life course assessment of SBP may identify differences in blood pressure between WT and P0 offspring at later postnatal ages.

暴露于SC在宫内显著增加了后代的SBP。之前的研究已经表明，P0雄性后代的出生体重较限制，表现出早期生活期的追赶性生长，但继续比WT同窝小鼠轻7%。虽然P0胎儿在接近胎龄时的生长受到限制，但在8或13周龄时我们没有看到P0和WT后代从生理盐水处理的妊娠中产生的不同。一个生活阶段的血压评估可能会在后代的后期后natal年龄发现不同的血压。
growth-restricted and normal pregnancies. There was an average increase in SBP of ~24 mmHg in mice exposed to SC in utero. Our data are in accord with that of Mills et al. (28), who demonstrated that eNOS−/− offspring exposed to SC in utero had increased SBP in later life. In contrast, this same study saw no increase in SBP in WT offspring after antenatal exposure to SC, different from our findings. The disparity between the effects of SC exposure in utero in control (WT) mice may be a consequence of the differences in dosing regimen or may simply be due to the age at which blood pressure was measured i.e., 13 wk vs. 20 wk.

**Mesenteric Artery Function**

One potential mediator of increased SBP is increased peripheral vascular resistance, which might be caused by aberrant vascular function of resistance blood vessels (5, 40). Such dysfunction can be defined as an enhanced response to contractile agents and/or reduced endothelial-dependent and independent relaxation (3, 37). However, there was no consistent dysfunction in contraction or relaxation responses that could underlie the increase in SBP in either WT or P0 offspring of SC-treated dams. This is in contrast to a study where changes in SBP in offspring following exposure to SC in pregnant eNOS−/− mice were associated with an increase in U46619-induced contraction of mesenteric arteries of male mice at 21 wk of age (28).

**Conclusions**

We have, for the first time, demonstrated that exposure to SC in utero is associated with raised SBP in offspring independent of sex or genotype. Although we acknowledge the clinical importance of these data, there are a number of limitations which should be addressed in future studies. We did not assess cardiovascular function or perform terminal cardiopulmonary analyses in offspring, which could have provided a mechanism for raised SBP associated with SC. Furthermore, we were unable to assess mechanisms associated with differences in body weight in female offspring that were exposed to SC in utero. The translational impact of our work could be improved with these data, and any associations with the STRIDER trial must therefore take into account these limitations.

There have been a number of experimental and clinical studies assessing the effects of antenatal SC treatment during pregnancy. A systematic review of experimental and clinical studies of SC for FGR (44) highlighted only one study where maternal administration of SC had detrimental effects on fetal well-being [a decrease in fetal weight (33)]. However, a later study in the SUAL sheep model of FGR revealed that SC led to reduced uterine artery blood flow in both SUAL and control ewes associated with hypotension, tachycardia, and reduced PO2 in fetuses from both th control and the SUAL ewes (27). The focus of previous studies has been to assess SC effects on the mother, fetus, and in some cases neonate, but only one study (28) has examined the effects of antenatal SC in the long term.

The findings of this study must be placed into the context of the recently published STRIDER clinical trials (18, 41) assessing the impact of SC in severe early onset FGR. There were no effects of SC treatment on the prolongation of pregnancy, birth weight, or rates of fetal death in two cohorts of women with dismal prognosis (18, 41). There are, however, potentially concerning indications that SC led to reductions in fetal ductus venous blood flow (41), and recently, 11 neonatal deaths led to the cessation of the Dutch STRIDER trial (19). Neonatal follow-up (<2 yr) will be assessed in children from SC-treated pregnancies; however, the data provided herein and elsewhere (28) suggest that it is very important to continue follow-up into at least early adulthood.

In the present study, and in line with the published outcomes of the various STRIDER trials, we have demonstrated that there was no effect of SC on fetal weight or fetal viability near term. We have also demonstrated that maternal SC treatment elevated SBP in the offspring of these pregnancies as early as 8 wk of age in the mouse, irrespective of genotype or sex, through as yet unidentified mechanisms. Combined, the outcomes of our study and that of Mills et al. (28), and in line with

Table 1. Offspring organ allometry from saline- and sildenafil citrate-treated pregnancies

| Offspring ID | Heart Weight | Average Kidney Weight | Spleen Weight | Lung Weight | Brain Weight |
|-------------|--------------|------------------------|--------------|------------|-------------|
|             | Value        | n                      | Value        | n          | Value        | n          |
| Saline      |              |                        |              |            |              |            |
| Male        |              |                        |              |            |              |            |
| WT          | 0.51 [0.41–0.62] | 11 | 1.16 [1.03–1.36]** | 11 | 0.23 [0.20–0.29]** | 11 | 0.46 [0.43–0.54]***** | 10 | 1.28 [1.19–1.56]**** | 11 |
| P0          | 0.43 [0.39–0.66]* | 7 | 1.15 [1.03–1.29] * | 7 | 0.23 [0.19–0.27]* | 7 | 0.46 [0.44–0.58]* | 7 | 1.31 [1.20–1.40] | 6 |
| Female      |              |                        |              |            |              |            |
| WT          | 0.51 [0.42–0.80] | 18 | 1.04 [0.94–1.20]** | 18 | 0.31 [0.20–0.37]** | 18 | 0.57 [0.52–0.69]*** | 16 | 1.73 [1.54–2.00]**** | 18 |
| P0          | 0.51 [0.46–0.52] | 4 | 1.01 [0.97–1.21] | 5 | 0.35 [0.26–0.37]* | 4 | 0.54 [0.47–0.57] | 3 | 1.61 [1.51–1.88] | 4 |
| Sildenafil citrate (10 mg/kg) |              |                        |              |            |              |            |
| Male        |              |                        |              |            |              |            |
| WT          | 0.49 [0.38–0.71] | 19 | 1.11 [0.97–1.38] | 16 | 0.23 [0.20–0.30] | 16 | 0.52 [0.41–0.63] | 16 | 1.39 [1.22–1.66] | 16 |
| P0          | 0.51 [0.43–0.72]* | 9 | 1.17 [1.01–1.23] * | 6 | 0.23 [0.21–0.28] | 6 | 0.43 [0.39–0.56]* | 6 | 1.33 [1.26–1.43] | 6 |
| Female      |              |                        |              |            |              |            |
| WT          | 0.47 [0.37–0.67] | 16 | 1.05 [0.90–1.34] | 15 | 0.33 [0.29–0.38] | 15 | 0.54 [0.44–0.70] | 15 | 1.65 [1.34–1.83] | 15 |
| P0          | 0.47 [0.44–0.63] | 7 | 0.97 [0.87–1.05] | 4 | 0.32 [0.31–0.36] | 4 | 0.53 [0.44–0.54] | 4 | 1.61 [1.41–1.80] | 4 |

Values are medians [min–max] from 11 litters. P0, placental-specific insulin-like growth factor 2 knockout; WT, wild type. Organ wet weights measured at wk 14 and presented as %body weight. Organ weights were assessed for effect of genotype and sex within saline-treated groups by using the Kruskal-Wallis statistical test with identical letters denoting significance between groups using Dunn’s multiple comparisons post hoc test. A Mann-Whitney U-test was used to assess the effect of treatment between two groups; e.g., WT males from saline-treated pregnancy vs. WT males from sildenafil citrate-treated pregnancy. *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001 for post hoc test.
the findings of the STRIDER consortium (17, 18, 41), do not support maternal SC treatment as a safe or effective treatment for human FGR.

This study highlights the importance of assessing both the short and long-term consequences of therapeutics administered during pregnancy. Such data from pre-clinical models are crucial in affording a more informed choice for the obstetrician and patient on the potential short- and long-term risk vs. benefits of treatment in utero.

ACKNOWLEDGMENTS

We thank the staff at The University of Manchester Biological Sciences Facility for assistance during the project.

GRANTS

This study was supported by The Medical Research Council Program (MRC) Grant G0802770. M. R. Dilworth is supported by an MRC Career Development Award Grant MR/K024442/1.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

L.J.R., E.C.C., C.P.S., P.N.B., S.L.G., M.W., and M.R.D. conceived and designed research; L.J.R., E.C., E.T., M.W., and M.R.D. performed experiments; L.J.R. and E.T. analyzed data; L.J.R. interpreted results of experiments; L.J.R., E.C., E.T., M.W., and M.R.D. edited and revised manuscript; L.J.R., E.C.C., E.C., C.P.S., P.N.B., E.T., S.L.G., M.W., and M.R.D. approved final version of manuscript.

REFERENCES

1. Ashworth A. Effects of intrauterine growth retardation on mortality and morbidity in infants and young children. *Eur J Clin Nutr* 52, Suppl 1: S34–S41, 1998.
2. Barker DJ, Eriksson JG, Forsén T, Osmond C. Fetal origins of adult disease: strength of effects and biological basis. *Int J Epidemiol* 31: 1235–1239, 2002. doi:10.1093/ije/31.6.1235.
3. Brawley L, Itoh S, Torrens C, Barker A, Bertram C, Poston L, Cottrell EC, Sibley CP. Dietary protein restriction in pregnancy induces hypertension and vascular defects in rat male offspring. *Pediastr Res* 53: 83–90, 2003. doi:10.1203/01.PDR.0000065731.00639.02.
4. Bukowski R, Hansen NI, Willinger M, Reddy UM, Parker CB, Pinar FM, Mikaelsson MA, Constância M, Dent CL, Wilkinson LS, Humby T. From pre-clinical studies to clinical trials: proving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 11: e1001633, 2013. doi:10.1371/journal.pmbi.1001633.
5. Christensen KL, Mulvany MJ. Mesenteric arcade arteries contribute substantially to vascular resistance in conscious rats. *J Vasc Res* 30: 73–79, 1993. doi:10.1159/000015897.
6. Constância M, Dean W, Lopes S, Moore T, Kelsey G, Reik W. Deletion of a silencer element in Igf2 results in loss of imprinting independent of H19. *Nat Genet* 26: 203–206, 2000. doi:10.1038/79930.
7. Constância M, Hemberger M, Hughes J, Dean W, Ferguson-Smith A, Fundele R, Stewart F, Kelsey G, Fowden A, Sibley C, Reik W. Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature* 417: 945–948, 2002. doi:10.1038/nature00869.
8. Cottrell EC, Sibley CP. From pre-clinical studies to clinical trials: Generation of novel therapies for pregnancy complications. *Int J Mol Sci* 16: 12907–12924, 2015. doi:10.3390/ijms160612907.
9. de Jong F, Monuteaux MC, van Elburg RM, Gillman MW, Belfort MB. Systematic review and meta-analysis of perinatal birth and later systolic blood pressure. *Hypertension* 59: 226–234, 2012. doi:10.1161/HYPERTENSIONAHA.111.181784.
10. Dilworth MR, Andersson I, Renshall LJ, Cowley E, Baker P, Greenwood S, Sibley CP, Wareing M. Sildenafil citrate increases fetal weight in a mouse model of fetal growth restriction with a normal vascular phenotype. *PLoS One* 8: e77748, 2013. doi:10.1371/journal.pone.0077748.
11. Dilworth MR, Kusinski LC, Baker BC, Renshall LJ, Greenwood SL, Sibley CP, Wareing M. Defining fetal growth restriction in mice: a standardized and clinically relevant approach. *PLoS C 32: 914–916, 2011. doi:10.1016/j.placenta.2011.08.007.
12. Dilworth MR, Kusinski LC, Cowley E, Ward BS, Husain SM, Constância M, Sibley CP, Glazier JD. Placental-specific Igf2 knockout mice exhibit hypocacemia and adaptive changes in placental calcium transport. *Proc Natl Acad Sci USA* 107: 3894–3899, 2010. doi:10.1073/pnas.0911710107.
13. Figueras F, Gratacós E. Update on the diagnosis and classification of fetal growth restriction and proposal of a stage-based management protocol. *Fetal Diagn Ther* 36: 86–98, 2014. doi:10.1159/000357592.
14. Fisk NM, Atun R. Market failure and the poverty of new drugs in maternal health. *PLoS Med* 5: e22, 2008. doi:10.1371/journal.pmed.0050022.
15. Flenady V, Koopmans L, Middleton P, Fransen JF, Smith GC, Gibbons K, Cooyee M, Gordon A, Ellwood D, McIntyre HD, Frets R, Ezzati M. Major risk factors for stillbirth in high-income countries: a systematic review and meta-analysis. *Lancet* 377: 1331–1340, 2011. doi:10.1016/S0140-6736(10)61223-7.
16. Ganzvoort W, Allfirev Z, von Dadelszen P, Kenny L, Papageorghiou A, van Wassenber-Leemhuis A, Glud C, Mol BW, Baker PN. STRIDER: sildenafil therapy in dismal prognosis early-onset intrauterine growth restriction: a protocol for a systematic review with individual participant data and aggregate data meta-analysis and trial sequential analysis. *Syst Rev* 3: 23, 2014. doi:10.1186/2045-4033-3-23.
17. Groom KM, Ganzvoort W, Allfirev Z, Lim K, Papageorghiou AT. STRIDER Consortium. A comment on the safety and efficacy of sildenafil for fetal growth restriction (FGR): comment from the STRIDER Consortium. *Ultrasound Obstet Gynecol* 52: 295–296, 2018. doi:10.1002/uog.19186.
18. Groom KM, McCowan LM, Mackay LK, Lee AC, Gardener G, Unterscheider J, Sekar K, Dickinison JE, Muller P, Reid RA, Watson D, Welsh A, Marlow J, Walker SP, Hyett J, Morris J, Stone FR, Baker PN. STRIDER NZAuc: a multicentre randomised controlled trial of sildenafil therapy in early-onset fetal growth restriction. *BJOG* 126: 997–1006, 2019. doi:10.1111/1471-0528.15658.
19. Hawkes N. Trial of viagra for fetal growth restriction is halted after baby deaths. *BMJ* 362: k3247, 2018. doi:10.1136/bmj.k3247.
20. Huxley RR, Shiell AW, Law CM. The role of size at birth and postnatal catch-up growth in determining systolic blood pressure: a systematic review of the literature. *J Hypertens* 18: 815–831, 2000. doi:10.1097/00004872-200018070-00002.
21. Keijzer-Veen MG, Dülger A, Dekker FW, Nauta J, van der Heijden K, Carlo W, Tyson JE, Donovan EF, Shankaran S, Stevenson DK. Market failure and the poverty of new drugs in maternal health. *PLoS Med* 5: e22, 2008. doi:10.1371/journal.pmed.0050022.
22. Kussinski LC, Dilworth MR, Baker PN, Sibley CP, Wareing M, Glazier JD. System A activity and vascular function in the placental-specific Igf2 knockout mouse. *Placenta* 32: 871–876, 2011. doi:10.1016/j.placenta.2011.07.086.
23. Lemons JA, Bauer CR, Oh W, Korones SB, Papile L-A, Stoll BJ, Keel PR, Miller SL, Loose JM, Jenkin G, Wallace EM. The effects of sildenafil citrate (Viagra) on uterine blood flow and well being in the intrauterine growth-restricted fetus. *Am J Obstet Gynecol* 200: 102.e1–102.e7, 2009. doi:10.1016/j.ajog.2008.08.029.
28. Mills V, Plows JF, Zhao H, Oyston C, Vickers MH, Baker PN, Stanley JL. Effect of sildenafil citrate treatment in the eNOS knockout mouse model of fetal growth restriction on long-term cardiometabolic outcomes in male offspring. *Pharmacol Res* 137: 122–134, 2018. doi: 10.1016/j.phrs.2018.09.023.

29. Nair AB, Jacob S. A simple practice guide for dose conversion between animals and human. *J Basic Clin Pharm* 7: 27–31, 2016. doi: 10.4103/0976-0105.177703.

30. Oyston C, Stanley JL, Oliver MH, Bloomfield FH, Baker PN. Maternal administration of sildenafil citrate alters fetal and placental growth and fetal-placental vascular resistance in the growth-restricted ovine fetus. *Hypertension* 68: 760–767, 2016. doi: 10.1161/HYPERTENSIONAHA.116.07662.

31. Pellicer B, Herraiz S, Carbonell V, Morcillo E, Felipo V, Simón C, Pellicer A. Haemodynamic effects of long-term administration of sildenafil in normotensive pregnant and non-pregnant rats. *BJOG* 118: 615–623, 2011. doi: 10.1111/j.1471-0528.2010.02839.x.

32. Pettersson US, Waldén TB, Carlsson PO, Jansson L, Phillips M. Female mice are protected against high-fat diet induced metabolic syndrome and increase the regulatory T cell population in adipose tissue. *PLoS One* 7: e46057, 2012. doi: 10.1371/journal.pone.0046057.

33. Refuerzo JS, Sokol RJ, Aranda J, Hallak M, Hotra JW, Kruger M, Sorokin Y. Sildenafil citrate and fetal outcome in pregnant rats. *Fetal Diagn Ther* 21: 259–263, 2006. doi: 10.1159/000091352.

34. Renshall LJ, Dilworth MR, Greenwood SL, Sibley CP, Wareing M. In vitro assessment of mouse fetal abdominal aortic vascular function. *Am J Physiol Regul Integr Comp Physiol* 307: R746–R754, 2014. doi: 10.1152/ajpregu.00058.2014.

35. Samangaya RA, Mires G, Shennan A, Skillern J, Serra V, Morcillo E, Felipo V, Simón C, Pellicer A. Sildenafil citrate and fetal outcome in pregnant rats. *Fetal Diagn Ther* 21: 259–263, 2006. doi: 10.1159/000091352.

36. Sasser JM, Baylis C. Effects of sildenafil on maternal hemodynamics and fetal growth in normal rat pregnancy. *Am J Physiol Regul Integr Comp Physiol* 298: R433–R438, 2010. doi: 10.1152/ajpregu.00198.2009.

37. Sathishkumar K, Balakrishnan MP, Yallampalli C. Enhanced mesenteric arterial responsiveness to angiotensin II is androgen receptor-dependent in prena tally protein-restricted adult female rat offspring. *Biol Reprod* 92: 55, 2015. doi: 10.1095/biolreprod.114.126482.

38. Satterfield MC, Bazer FW, Spencer TE, Wu G. Sildenafil citrate treatment enhances amino acid availability in the conceptus and fetal growth in an ovine model of intrauterine growth restriction. *J Nutr* 140: 251–258, 2010. doi: 10.3945/jn.110.114678.

39. Scaffidi J, Mol BW, Keelan JA. The pregnant women as a drug orphan: a global survey of registered clinical trials of pharmacological interventions in pregnancy. *BJOG* 124: 132–140, 2017. doi: 10.1111/1471-0528.14151.

40. Schiffrin EL. Reactivity of small blood vessels in hypertension: relation with structural changes. State of the art lecture. *Hypertension* 19, Suppl. III–I19, 1992. doi: 10.1161/01.HYP.19.2.Suppl.III-a.

41. Sharp A, Cornforth C, Jackson R, Harrold J, Turner MA, Kenny LC, Baker PN, Johnston ED, Khalil A, von Dadelszen P, Papageorghiou AT, Alfirevic Z; STRIDER group. Maternal sildenafil for severe fetal growth restriction (STRIDER): a multicentre, randomised, placebo-controlled, double-blind trial. *Lancet Child Adolesc Health* 2: 93–102, 2018. doi: 10.1016/S2355-4642(17)30173-6.

42. Sibley CP, Coan PM, Ferguson-Smith AC, Dean W, Hughes J, Smith P, Reik W, Burton GJ, Fowden AL, Constância M. Placental-specific insulin-like growth factor 2 (Igf2) regulates the directional exchange characteristics of the mouse placenta. *Proc Natl Acad Sci USA* 101: 8204–8208, 2004. doi: 10.1073/pnas.0402508101.

43. Stanley JL, Andersson LJ, Poudel R, Rueda-Clausen CF, Sibley CP, Davidge ST, Baker PN. Sildenafil citrate rescues fetal growth in the catechol-O-methyl transferase knockout mouse model. *Hypertension* 59: 1021–1028, 2012. doi: 10.1161/HYPERTENSIONAHA.111.186270.

44. Villanueva-García D, Mota-Rojas D, Hernández-González R, Sánchez-Aparicio P, Alonso-Spilsbury M, Trujillo-Ortega ME, Necochea RR, Nava-Ocampo AA. A systematic review of experimental and clinical studies of sildenafil citrate for intrauterine growth restriction and pre-term labour. *J Obstet Gynaecol* 27: 255–259, 2007. doi: 10.1080/0144361070194978.

45. von Dadelszen P, Dwinell S, Magee LA, Carleton BC, Gruslin A, Lee B, Lim KI, Liston RM, Miller SP, Rurak D, Sherlock RL, Skoll MA, Wareing MM, Baker PN; Research into Advanced Fetal Diagnosis and Therapy (RAFT) Group. Sildenafil citrate therapy for severe early-onset intrauterine growth restriction and pre-term birth. *BJOG* 118: 624–628, 2011. doi: 10.1111/j.1471-0528.2010.02879.x.

46. Walker DK, Ackland MJ, James GC, Muirhead GJ, Rance DJ, Wastall P, Wright PA. Pharmacokinetics and metabolism of sildenafil in mouse, rat, rabbit, dog and man. *Xenobiotica* 29: 297–310, 1999. doi: 10.1080/004982599238687.

47. Wareing MM, Myers JE, O’Hara M, Baker PN. Sildenafil citrate (Viagra) enhances vasodilatation in fetal growth restriction. *J Clin Endocrinol Metab* 90: 2550–2555, 2005. doi: 10.1210/jc.2004-1831.

48. Webb JF. Canadian thalidomide experience. *Can Med Assoc J* 89: 987–992, 1963.

49. Witjes BC, Ahsmann MJ, van der Nagel BC, Tibboel D, Mathot RA. Simultaneous assay of sildenafil and desmethylsildenafil in neonatal plasma by ultra-performance liquid chromatography-tandem mass spectrometry. *Biomed Chromatogr* 24: 180–185, 2010.