Sildenafil Citrate Rescues Fetal Growth in the Catechol-O-Methyl Transferase Knockout Mouse Model

Joanna L. Stanley, Irene J. Andersson, Rajan Poudel, Christian F. Rueda-Clausen, Colin P. Sibley, Sandra T. Davidge, Philip N. Baker

Abstract—Preeclampsia and fetal growth restriction are responsible for the majority of maternal and perinatal morbidity and mortality associated with complicated pregnancies. Although their etiologies are complex and multifactorial, both are associated with increased uterine artery resistance. Sildenafil citrate is able to rescue the dysfunction observed ex vivo in uterine arteries of women with preeclampsia. The ability of sildenafil citrate to increase uterine artery vasodilation, thereby decreasing uterine artery resistance and, hence, ameliorated preeclampsia and fetal growth restriction, was tested in a mouse model of preeclampsia, the catechol-O-methyl transferase knockout mouse (COMT\textsuperscript{−/−}). COMT\textsuperscript{−/−} and C57BL/6J mice were treated (0.2 mg/mL in drinking water, n=6–12) from gestational day 12.5 to 18.5. Measures of pup growth, including body weight, crown/rump length, and abdominal circumference, were reduced in COMT\textsuperscript{−/−} mice; this was normalized after treatment with Sildenafil. COMT\textsuperscript{−/−} mice also demonstrated abnormal umbilical Doppler waveforms, including reverse arterial blood flow velocity. This was normalized after treatment with Sildenafil. Abnormal uterine artery Doppler waveforms were not demonstrated in COMT\textsuperscript{−/−} mice, although ex vivo responses of uterine arteries to phenylephrine were increased; moreover, treatment with Sildenafil did improve ex vivo sensitivity to an endothelium-dependent vasodilator. The data presented here demonstrate that Sildenafil can rescue pup growth and improve abnormal umbilical Doppler waveforms, providing support for a potential new therapeutic strategy targeting fetal growth restriction. (Hypertension. 2012;59:1021-1028.) ● Online Data Supplement

Key Words: preeclampsia ■ fetal growth restriction ■ mice ■ uterine artery ■ umbilical artery

Preeclampsia (PE) and fetal growth restriction (FGR) are pregnancy-specific disorders that complicate ≤10% of all pregnancies.\textsuperscript{1} Together they are responsible for the majority of maternal and fetal morbidity and mortality associated with complicated pregnancies.\textsuperscript{2,3} PE, characterized by the onset of hypertension and proteinuria during pregnancy, is a leading cause of maternal mortality.\textsuperscript{4,5} It is also associated with an increased risk of stillbirth, sudden infant death, and FGR.\textsuperscript{1} FGR, which is defined as a fetus that fails to reach its genetic growth later in life.\textsuperscript{7} There are currently only limited treatment options available for PE and none for FGR; an effective therapeutic strategy would have major clinical significance.

The etiology of PE and FGR is complex and as yet poorly understood; common to both conditions, however, is an increase in uterine artery resistance.\textsuperscript{8} This leads to reduced uteroplacental perfusion\textsuperscript{9,10}, in turn, this may impact fetoplacental vascular development and result in increased resistance in the umbilical circulation.\textsuperscript{11} In addition, hypoperfusion of the placenta also causes an increase in the production of reactive oxygen species, such as superoxide anions, which may reduce vasodilation further because of the scavenging of NO. It has been demonstrated previously that exposure of placental arteries to reactive oxygen species, such as superoxide, increases contractility,\textsuperscript{12} suggesting that elevated oxidative stress may play a role in mediating the pathophysiology of PE and FGR. It is possible, therefore, that treatments that increase uteroplacental perfusion may ameliorate the symptoms of PE and rescue fetal growth.

The aim of this study was to test this hypothesis using a mouse model, the catechol-O-methyltransferase knockout mouse, referred to as COMT\textsuperscript{−/−} throughout. COMT\textsuperscript{−/−} mice have been shown previously to exhibit a PE-like phenotype, which includes hypertension near the end of pregnancy and proteinuria.\textsuperscript{13}

The treatment tested in this study was Sildenafil citrate, a phosphodiesterase-5 (PDE5) inhibitor; PDE5 is a down-
stream modulator of the vasodilator NO. NO plays a vital role in mediating the cardiovascular adaptations that occur during pregnancy. These adaptations include enhanced vasodilatation, which facilitates adequate uteroplacental perfusion and is observed in both humans and mice. This adaptation appears to be missing in arteries taken from women with PE. In addition, impaired endothelium-dependent vasodilatation of myometrial arteries taken from women with PE and FGR is improved, ex vivo, after PDE5 inhibition. There is, therefore, evidence that vasodilatation of the uterine circulation is impaired in PE and FGR, which may be attributed in part to reduced NO-mediated vasodilatation. In addition, these studies suggest that PDE5 inhibition by Sildenafil may be able to improve uterine artery vasodilatation, thus mediating improved uteroplacental perfusion and increased fetal growth. Therefore, this study tested the hypothesis that treatment of pregnant COMT−/− mice with Sildenafil citrate will improve uteroplacental perfusion, ameliorate the PE-like symptoms, and improve fetal growth.

Methods

All of the protocols were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care and conformed to the Guide for the Care and Use of Laboratory Animals (copyright 1996, National Academy of Science).

Animals and Treatments

Female COMT−/− (obtained under a material transfer agreement from Prof J Gogos, Columbia University) and C57Bl6/J mice (purchased from Jackson Laboratories) of 8 to 12 weeks of age were mated nightly with males of corresponding genotype. The day a plug was detected was denoted as gestational day (GD) 0.5. Sildenafil was administered from GD 12.5, which is acknowledged as the day on which placental development is equivalent to the end of the first trimester in the human and the time at which uteroplacental circulation is fully open and functional, to GD 18.5 (term is GD 19.5). A total of 6 treated and 6 untreated COMT−/− mice were used and 12 untreated C57Bl6/J mice were used. All of the protocols were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care and conformed to the Guide for the Care and Use of Laboratory Animals (copyright 1996, National Academy of Science).

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Blood Pressure and Proteinuria

Blood pressure was measured using the tail-cuff method (Ishihara Life Science, Woodland Hills, CA). Before mating, mice were placed in restraint tubes for 5 minutes on 3 consecutive days. Nonpregnant blood pressure was then recorded using tail-cuff plethysmography. Readings were also made at GDs 10.5 and 17.5.

Urine was collected at GD 18.5 and stored at −80°C. Samples were then assayed to assess albumin concentration (AssayPro, St Charles, MO), as well as creatinine concentration (Cayman Chemical Company, Ann Arbor, MI).

Ultrasound Biomicroscopy

Uterine artery, umbilical artery, and vein blood flow velocities were assessed at GDs 11.5 and 17.5 using an ultrasound biomicroscope (model Vevo 770, VisualSonic, Toronto, Ontario, Canada). Both left and right uterine arteries and the umbilical artery from ≥2 fetuses were assessed (please see the online-only Data Supplement).

Fetal and Placental Measurements

Mice were culled on GD 18.5 and pups and placentas dissected out. Pups from the right uterine horn were blotted and weighed and fetal crown to rump length and abdominal circumference determined. The gross anatomy of the pups was also examined. Placentas were blotted dry, weighed, then dried at 40°C, and dry weight recorded. Uterine arteries from the right horn were dissected clean in cold physiological saline solution (in mmol/L; 10 000 HEPES, 142.000 NaCl, 4.700 KCl, 1.200 MgSO4, 1.600 CaCl2, 1.180 KH2PO4, 5.500 glucose, 0.034 EDTA; pH 7.4) and mounted on a wire myograph for assessment of vascular function.

Ex Vivo Vascular Function

Uterine arteries were collected at GD 18.5. Four segments, taken from the main loop uterine artery of each mouse, were used; vascular function was investigated by determining vasoconstrictor responses to phenylephrine, endothelium-dependent relaxation in response to methacholine, and endothelium-independent relaxation to the NO donor sodium nitroprusside (please see the online-only Data Supplement).

Statistical Analysis

All of the normally distributed data are expressed as mean±SEM and were compared using the Student’s t test or 2-way ANOVA that included genotype and treatment as sources of variation followed by Bonferroni post hoc test. A P value <0.05 was considered statistically significant. Sigmoidal curve fitting was performed on wire myography concentration-response curve data using GraphPad Prism 5.0 software; curves were then used to determine either EC50 or EC80 values.

Results

Blood Pressure and Proteinuria

Systolic blood pressure was significantly increased in COMT−/− mice compared with C57Bl6/J mice in nonpregnant animals (127.4±2.8 versus 119.3±2.6 mmHg; P<0.05). This increase was also observed at GD 10.5 (126.8±3.1 versus 118.1±2.2 mmHg; P<0.05). In untreated mice, there was no difference in systolic blood pressure between COMT−/− and C57Bl6/J mice at GD 17.5, and treatment with Sildenafil had no effect (please see Figure S1 in the online-only Data Supplement).

Proteinuria was assessed by calculating the albumin:creatinine ratio at GD 18.5. There was a significant effect of genotype on this measurement (Figure 1; P<0.01). The ratio was significantly increased in COMT−/− mice treated with...
Sildenafil compared with similarly treated C57BL/6J mice (Figure 1; *P*<0.05). Treatment with Sildenafil had no effect on proteinuria.

**Fetal and Placental Measurements**

Pup weight was significantly decreased in COMT/−/− compared with control C57BL/6J mice (Figure 2A; *P*<0.01). This was highlighted further when pup weight was fitted to a Gaussian distribution (Figure 2B). Treatment with Sildenafil significantly increased pup weight in COMT/−/− mice (Figure 2A; *P*<0.05). Similarly, crown-to-rump length (Figure 2C) and abdominal circumference (Figure 2D) were significantly reduced in pups from COMT/−/− compared with control mice (*P*<0.01 and *P*<0.001, respectively). Again, treatment with Sildenafil significantly increased both measurements (*P*<0.05).

Placental wet and dry weight were significantly increased in tissue from COMT/−/− mice compared with controls (*P*<0.01 and *P*<0.001, respectively; please see Figure S2A and S2B, respectively); this was not altered after treatment with Sildenafil. Pup weight:placenta weight ratio was significantly decreased in COMT/−/− mice (Figure 3; *P*<0.001); again, this was not altered by Sildenafil treatment. Pups from mice treated with Sildenafil were examined for gross abnormalities; none were observed in either group.

**Uterine and Umbilical Artery Blood Flow Velocity**

The hemodynamic and waveform parameters, as observed at GD 17.5, are detailed in the Table (please see the online Data Supplement; details of parameters measured at GD 11.5 are shown in Table S1). There were no significant differences in uterine artery waveform parameters between C57BL/6J and COMT/−/− mice at either gestational age.

There were, however, significant changes in umbilical artery blood flow velocity measurements. Minimum umbilical artery velocity and, consequently, echographic markers of peripheral vascular resistance, such as resistance index and frequency.
Pulsatility index were significantly increased in COMT<sup>−/−</sup> mice at GD 11.5 (resistance index) and 17.5 (resistance index and pulsatility index; P < 0.05); interestingly, all of the hemodynamic parameters were normalized after treatment with Sildenafil (Figure 4A and 4B; P < 0.05). In addition, reverse umbilical artery blood flow velocity was noted in COMT<sup>−/−</sup> mice at GD 17.5; again, this finding was not present after treatment with Sildenafil (Figure 4C).

Ex Vivo Vascular Function

Uterine arteries from COMT<sup>−/−</sup> mice exhibited increased maximal constriction in response to phenylephrine compared with arteries from control C57BL/6J mice (Figure 5A; P < 0.05). After treatment with Sildenafil, maximal constriction of uterine arteries from COMT<sup>−/−</sup> mice was normalized to that of control mice (Figure 5B).

There were no differences in maximal relaxation or sensitivity to methacholine in uterine arteries from COMT<sup>−/−</sup> and C57BL/6J mice (Figure 5C). Treatment with Sildenafil increased sensitivity to methacholine in arteries from both groups of mice (Figure 5D; P < 0.05), although there was no difference in the maximal relaxation achieved. There were no differences in relaxation in response to the NO donor sodium nitroprusside between uterine arteries from C57BL/6J and COMT<sup>−/−</sup> mice (data not shown).

Discussion

The data presented in this study demonstrate that treatment with Sildenafil was able to rescue fetal growth in the COMT<sup>−/−</sup> mouse model. Sildenafil therapy was associated with enhanced uterine artery vasodilation and reduced placental resistance.

There has been limited investigation of Sildenafil as a putative therapy for PE or FGR. A small, randomized, controlled trial that investigated the ability of PDE5 inhibitors to ameliorate the symptoms of PE did not demonstrate any...
benefit; however, plasma concentrations did not reach therapeutic levels, and the study was discontinued before recruitment of the planned sample size could be completed. More recently, a controlled clinical trial in which women with severe early onset FGR were offered Sildenafil therapy reported that Sildenafil treatment was associated with an increase in fetal abdominal circumference. The recent development of animal models that display a PE- and/or FGR-like phenotype provides a tool that allows a more thorough investigation of the potential benefits of Sildenafil therapy for these conditions.

The PE-like phenotype of the pregnant COMT knockout mouse was first described by Kanasaki et al. They observed a significant increase in systolic blood pressure compared with wild-type controls at GD 17.5, as well as a significant increase in proteinuria. Although aspects of the PE-like phenotype were again demonstrated in this study, the phenotype differed from that observed previously; an increase in systolic blood pressure in mice was demonstrated compared with C57BL/6J controls in nonpregnant mice and at GD 10.5 (which was not observed in the previous study). This increase, however, was no longer present at GD 17.5. Systolic blood pressure was measured using the tail-cuff method in both studies. Although this technique is well described, the values obtained may be more variable than those obtained using telemetry and may partially explain the differing results observed in this study. The data presented in this study for control (C57BL/6J) mice, however, are comparable with those obtained previously using telemetry. The increase in proteinuria observed was similar in both studies. There was no effect of Sildenafil on either systolic blood pressure or proteinuria in COMT knockout mice. The lack of effect of Sildenafil on systolic blood pressure was consistent with previous reports in mice that have demonstrated that Sildenafil does not affect blood pressure.

A small but significant decrease in pup growth was noted in this study in COMT knockout compared with C57BL/6J mice; this again differs from the previous study but may be attributed to the difference in gestational age at which the measurements were made. Sildenafil significantly increased all of the measures of pup growth used in COMT knockout mice (weight, crown-to-rump length, and abdominal circumference). The significant increase in placental weight observed in COMT knockout mice, along with a decreased pup weight:placental weight ratio, suggests that inadequate placental nutrient exchange capacity (“placental insufficiency”) may be one cause of the growth restric-
tion noted. This, unlike pup growth, however, was not improved after treatment with Sildenafil.

A number of clinical studies have been performed to determine the ability of both uterine and umbilical artery Doppler indices to predict the development of both FGR and PE. The significance of abnormal uterine artery Doppler findings is somewhat contentious; changes such as abnormal uterine artery flow waveform ratios, the presence of a diastolic notch, or increased resistance or pulsatility indices have all been associated with the development of FGR but have a low-to-moderate predictive value.25,26 In contrast, abnormal umbilical artery and vein Doppler waveforms are associated with a greater risk of FGR. Increased umbilical artery resistance is associated with decreased birth weight, as well as increased placental resistance27 and changes in tertiary stem villi vessel morphometry.28 In addition, cases with more severe abnormalities, such as absent or reverse end-diastolic umbilical artery flow, develop severe FGR and demonstrate significant placental pathology, such as a reduction in branching of terminal stem villi, which are vital for gas exchange.29 Abnormal umbilical artery Doppler waveforms in cases of FGR are also associated with both hemorrhagic and ischemic placental lesions.30 Abnormalities of the umbilical vein Doppler waveform are observed later than those in the umbilical artery and suggest a failure of the fetal circulation to compensate, that is, fetal cardiac dysfunction.31–33

The observations made in COMT−/− mice in this study, including reverse flow and significantly reduced minimum umbilical artery blood flow velocity, as well as abnormalities in the umbilical vein Doppler waveform, suggest that the growth restriction observed is associated with increased resistance to flow in the fetoplacental circulation. A previous study, in which the COMT−/− mouse was identified as a model of PE, observed that placentas from these mice demonstrated an increase in the antiangiogenic factor soluble Fms-like tyrosine kinase 1.13 A reduction in angiogenesis may be one mechanism by which placental resistance is increased in this model. The normalization of pup growth, consequent on Sildenafil therapy, was accompanied by increases in umbilical artery and vein blood flow velocities, consistent with a reduction in placental resistance.

Although changes in the uterine artery Doppler waveform, such as an increased resistance index, are associated with FGR, no such changes were observed in this study. In line with this finding, ex vivo examination of uterine artery

Figure 5. Sildenafil normalizes vasoconstriction and increases sensitivity to the endothelium-dependent vasodilator methacholine. Maximal constriction of uterine arteries from catechol-O-methyl transferase knockout mouse (COMT−/−) mice (A and B) was significantly increased; this was normalized to that of control mice after treatment with Sildenafil. There was no difference in endothelium-dependent vasodilation of uterine arteries from COMT−/− and C57BL/6J mice (C). There was a significant effect of treatment with Sildenafil on sensitivity to methacholine (D). Mean±SEM, n=6; *P<0.05 for the respective source of variation (2-way ANOVA), †P<0.05 vs untreated animals of the same genotype (Bonferroni post hoc test), ■, untreated; □, Sildenafil treated.
endothelial function demonstrated that endothelium-dependent relaxation did not differ between C57BL/6J and COMT+/- mice. Although maximal constriction responses to phenylephrine were increased in uterine arteries of COMT+/- mice, the pregnancy adaptation(13), which enables full relaxation of the murine uterine artery, was unaffected. Treatment of both groups of mice with Sildenafil significantly increased the sensitivity of the response to the endothelium-dependent vasodilator methacholine. It should be acknowledged that uterine loop arteries were examined in this study, and that the site of resistance in this vascular bed is the branch arteries. Kusinski et al, however, compared both the contractile (in response to phenylephrine) and endothelium-dependent vasodilator properties of uterine loop and branch arteries from pregnant C57BL/6J mice and determined that there were no significant differences in the maximum contractile or relaxation response between the 2 types of vessel. It is known that reduced uteroplacental perfusion is associated with abnormal fetoplacental vascular development and increased resistance in the umbilical circulation37,38; increased placental perfusion as a consequence of increased uterine artery dilation may, therefore, have contributed to the reduced placental resistance observed after treatment with Sildenafil citrate.

Perspectives

Using the COMT+/- mouse model, it was demonstrated that reduced pup growth was associated with abnormal umbilical Doppler waveforms. In addition, it was found that both pup growth and umbilical Doppler waveforms were normalized after treatment with Sildenafil citrate; there is evidence that the effects of Sildenafil were mediated by a reduction in placental resistance. This important study provides crucial evidence to support the further research and development of a potential new therapy for FGR.

Acknowledgments

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Disclosures

None.

References


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SILDENAFIL CITRATE RESCUES FETAL GROWTH IN THE
COMT<sup>−/−</sup> MOUSE MODEL

Online Data Supplement

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Expanded Methods Section

Ultrasound biomicroscopy
Both uterine artery and umbilical artery and vein blood flow velocity was assessed at GD 11.5 and 17.5 of gestation. Both left and right uterine artery, and the umbilical artery from at least two fetuses were assessed. Before the procedure, mice were anaesthetized with isoflurane (3%) in air. Maternal heart rate, respiratory rate and rectal temperature were continuously monitored and anesthetic concentration was adjusted (~0.5 to 1.5%) to maintain a constant maternal heart rate of 550 ± 50 bpm and a respiratory rate of 120 ± 20 cpm. Heating was adjusted to maintain rectal temperature between 36° and 38°C. Hair was removed from the abdomen by shaving followed by a chemical hair remover. Pre-warmed gel was used as an ultrasound coupling medium. Mice were then imaged transcutaneously using an ultrasound biomicroscope (model Vevo 770, VisualSonics®, Toronto, ON, Canada) and a 30 MHz transducer operating at 100 frames/s, as previously described (35). In Doppler mode, the high-pass filter was set at 6 Hz and the pulse repetition frequency was set between 4 and 48 kHz. A 0.2 to 0.5 mm pulsed Doppler gate was used, and the angle between the Doppler beam and the vessel was <30°. Images were recorded for offline analysis. Doppler waveforms were obtained in both uterine arteries near the lateral-inferior margin of the utero-cervical junction close to the iliac artery on each side. Peak systolic velocity (PSV) and end diastolic velocity (EDV) were measured from at least 3 consecutive cardiac cycles that were not affected by motion caused by maternal breathing and the results were averaged. The pulsatility index (PI) and the resistive index (RI) were used as pulsed-wave Doppler measurements of downstream uterine and umbilical artery resistance and were calculated as PI=(PSV−MDV)/MV and RI=(PSV−MDV)/PSV where PSV=peak systolic velocity, DV=minimum diastolic velocity, and MV=mean velocity (time averaged velocity); when MDV<0 a velocity of 0.1 mm/s was used for calculation of PI and RI.

Ex vivo vascular function
Four uterine arteries segments were mounted in a wire myograph (610M, Danish Myo Technology, Aarhus, Denmark) using 25µm tungsten wire and warmed to 37°C for 20 minutes before they were normalized according to standard procedures. The vessels were then stimulated with phenylephrine (Phe, 10µmol/L) twice, with a 15-minute wash in-between. Methacholine (MCh, 10µmol/L) was added at the end of the second Phe-induced contraction to determine the integrity of the endothelium. A Phe dose-response curve (0.1nmol/L to 10µmol/L in nine steps) was performed, followed by careful washing. The vessels were then pre-constricted with Phe (at the EC80 concentration calculated following the Phe dose-response curve) and the endothelium-dependent relaxation response was tested by a cumulative dose-response curve to MCh (0.1 nmol/L to 10 µmol/L in nine steps). Finally, a dose-response curve to sodium nitroprusside (SNP, 0.1 nmol/L to 10 µmol/L in eight steps) was performed to validate endothelium-independent NO-mediated smooth muscle relaxation. All chemicals were obtained from Sigma (St Louis, MO, USA).
Table S1. Hemodynamic parameters of uterine and umbilical vasculature evaluated by ultrasound at gestational day 11.5.

<table>
<thead>
<tr>
<th>Hemodynamic Parameters</th>
<th>C57BL/6J (n=5)</th>
<th>COMT&lt;sup&gt;-/-&lt;/sup&gt; (n=5)</th>
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<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
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<td>Maternal temperature (°C)</td>
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<tr>
<td>Maternal heart rate (bpm)</td>
<td>565.2</td>
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<td>Fetal heart rate (bpm)</td>
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<td>3.5</td>
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<td><strong>Uterine Artery</strong></td>
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<tr>
<td>VTI (cm)</td>
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<tr>
<td>Max vel (mm/s)</td>
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<tr>
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<tr>
<td>Min vel (mm/s)</td>
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<td>Max gradient (mmHg)</td>
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<td>Mean gradient (mmHg)</td>
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<td>PI</td>
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<td>RI</td>
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<td><strong>Umbilical Artery</strong></td>
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<td>VTI (cm)</td>
<td>1.046</td>
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<td>VTI (cm)</td>
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<td>Min vel (mm/s)</td>
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<td>Mean gradient (mmHg)</td>
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VTI: Velocity-time integral of Doppler signal, PI: Pulsatility index, RI: Resistance index, 
*: p<0.05 vs C57BL/6J, Mann Whitney test.
Figure S1. Systolic blood pressure was not affected by genotype or treatment in late gestation.
There was no difference in systolic blood pressure at GD 17.5 between C57BL/6J and COMT−/− mice. Treatment of both groups of mice with Sildenafil had no effect on systolic blood pressure.
Mean ± SEM, n=6-25; two-way ANOVA.
Figure S2. Placental wet and dry weight was increased in COMT−/− mice
Placental wet (A) and dry (B) weight was significantly increased in COMT−/− compared with C57BL/6J mice. This was not affected by treatment with Sildenafil.