Original Article

Evaluation of Sildenafil and Tadalafil for Reversing Constriction of Fetal Arteries in a Human Placenta Perfusion Model

Robert B. Walton, Luckey C. Reed, Sarah M. Estrada, Stacey S. Schmiedecke, Diana L. Villazana-Kretzer, Peter G. Napolitano, Nicholas Ieronimakis

Abstract —Fetal growth restriction resulting from reduced placental blood perfusion is a major cause of neonatal morbidity and mortality. Aside from intense surveillance and early delivery, there is no treatment for fetal growth restriction. A potential treatment associated with placental vasoconstriction is the class of PDE5 (phosphodiesterase type 5) inhibitors such as sildenafil, which is known to cross the placenta. In contrast, tadalafil, a more potent and selective PDE5 inhibitor has not been studied in pregnancy or experimental models of fetal growth restriction. Therefore, we compared the efficacy of these 2 PDE5 inhibitors for reversing vasoconstriction in an ex vivo human placental model and evaluating molecular and physiological responses. Sildenafil and tadalafil were infused into the intervillous space in a preconstricted human placental dual cotyledon, dual perfusion assay for the comparison of arteriole pressures and molecular indicators of drug inhibition. Results indicate a decrease arterial pressure with sildenafil citrate compared with controls, whereas tadalafil showed no difference. PDE5 and endothelial nitric oxide synthase activity were altered with sildenafil but not tadalafil. Sildenafil citrate improved preconstricted placental arterial perfusion in a human placental model, whereas tadalafil showed no response. It is possible that tadalafil did not cross the human placental barrier or was degraded by trophoblasts. This study supports human clinical trials exploring sildenafil as a potential treatment for improving fetal blood flow in fetal growth restriction associated with vasoconstriction. **(*Hypertension*. 2018;72:00-00. DOI: 10.1161/HYPERTENSIONAHA.117.10738.)** ● **Online Data Supplement**

Key Words: arterial pressure ■ gestational age ■ sildenafil ■ tadalafil ■ vasodilation

Early-onset fetal growth restriction at <32 weeks gestational age is associated with substantial neonatal morbidity and mortality.[1] The causes of fetal growth restriction include maternal, fetal, and placental factors, as well as the constitutionally small fetus. Pathological growth restriction is often associated with abnormal umbilical artery and ductus venous Doppler studies.[2] Maternal vascular disease, including hypertension and preeclampsia, accounts for as much as 30% of cases of fetal growth restriction. Hypertensive associated fetal growth restriction is often associated with abnormal placental growth or development, as well as uteroplacental insufficiency, in which there is inadequate blood flow to the fetus. Other causes of fetal growth restriction include chromosomal or genetic anomalies (20%), multiple gestations (3%), congenital anomalies, infection, malnutrition, smoking, medications, and other environmental toxins.[3]

Current management of fetal growth restriction consists of eliminating risk factors such as smoking, drug abuse, strenuous exercise as well as optimal management of the maternal disease. Follow-up consists of serial growth ultrasounds, umbilical artery and ductus venosus Doppler analysis, antepartum fetal testing, umbilical artery Doppler analysis, and early delivery for the compromised fetal status. Evidence suggests that early delivery for fetuses that demonstrate absent or reversal of umbilical artery end-diastolic flow improves fetal outcome.[4] Several meta-analyses have demonstrated marginal benefit in low-dose aspirin in the prevention of fetal growth restriction.[5–7] Currently, there are no significant treatments for pregnancies complicated by early-onset fetal growth restriction other than delivery of the compromised fetus.

One potential treatment for fetal growth restriction is the PDE5 (phosphodiesterase type 5) inhibitor. This drug class has been shown to improve blood flow in multiple disease processes associated with vasoconstriction such as male erectile dysfunction.[8] PDE5 inhibitors prevent cyclic guanosine monophosphate from being degraded, leading to increased endothelial nitric oxide-mediated vasodilation and improved blood flow. The possible benefit of PDE5 inhibitors in pregnancies complicated by preterm fetal growth restriction would be that they could potentially increase the uteroplacental circulation via vasodilation of the small arteries in the myometrium and in the placenta.

Received December 11, 2017; first decision December 28, 2017; revision accepted April 11, 2018.

From the Department of Obstetrics and Gynecology (R.B.W., L.C.R., S.M.E., S.S.S., D.L.V.-K., P.G.N.) and Department of Clinical Investigation (N.I.), Madigan Army Medical Center, Joint Base Lewis-McCord, Tacoma, WA.

The online-only Data Supplement is available with this article at http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.117.10738/-/DC1.

Correspondence to Nicholas Ieronimakis, Department of Clinical Investigation, Madigan Army Medical Center, 9040 Jackson Ave, Joint Base Lewis-McCord, Tacoma, WA 98431. E-mail nicholas.m.ieronimakis.civ@mail.mil

The only PDE5 inhibitor studied in pregnancy is sildenafil citrate. Sildenafil citrate is known to cause vasodilation of the pulmonary vasculature, and it has been used in a few case reports of pregnant women with pulmonary hypertension or Eisenmenger syndrome.[9–11] It is also efficacious in persistent pulmonary hypertension of the newborn.[12] Studies involving sildenafil citrate in pregnant humans have been limited but have not shown any teratogenic effects.[13] From a human study in the prevention of preeclampsia, levels of sildenafil citrate were detected in the umbilical cord blood, demonstrating that it does cross the placenta into the fetal circulation.[14] In another case, sildenafil citrate was used to improve the umbilical artery pulsatility index in a patient with an early fetal growth-restricted pregnancy and resulted in a prolongation of her pregnancy.[15] One randomized controlled trial of sildenafil citrate in early-onset fetal growth restriction demonstrated improved fetal growth in utero but no significant difference in neonatal outcomes.[16] Finally, the just-released results of another randomized controlled trial (STRIDER [Sildenafil Therapy in Dismal Prognosis Early-Onset Intrauterine Growth Restriction]) demonstrated no difference in delay in delivery or neonatal morbidity or mortality.[17]

Tadalafil, another PDE5 inhibitor, has superior pharmacokinetic and pharmacodynamics properties compared with sildenafil citrate. Importantly, there are 11 known classes of PDEs in humans that are found in different organ systems that have various effects including vasodilation. Besides inhibiting PDE5, all phosphodiesterase inhibitors are known to inhibit PDE1, PDE6, PDE10, and PDE11 to varying degrees. Sildenafil citrate is 24-fold more likely to inhibit PDE1 than tadalafil. The PDE1 enzyme is present in the myocardium, vascular smooth muscle, and brain. Inhibition of PDE1 may cause hypotension, which could lead to uteroplacental insufficiency. So, tadalafil is less likely to cause hypotension, which would be more desirable in cases of fetal growth restriction. Also, tadalafil is a more potent inhibitor of the PDE5 enzyme than sildenafil citrate (inhibitory concentration of 50% of 0.94–6.4 nmol\L versus 3.5–8.5 nmol\L, respectively). Finally, tadalafil has a longer half-life than sildenafil citrate, 17.5 hours versus 3 to 5, respectively, allowing for a more reliable steady state concentration of PDE5 inhibitor over the course of time. However, tadalafil has not been studied previously in either animal or human models of growth restriction.[18]

To study PDE5 inhibitors, we chose a dual cotyledon, dual perfusion human placental model, which was initially established by Glance et al[19,20] and further adapted by our research group.[21–25] This ex vivo model has the benefit of simulating physiological conditions of a human pregnancy. Other benefits include the ability to test drug passage across the placenta barrier, as well as the ability to perform histology and molecular analysis. To our knowledge, the effects of any PDE5 inhibitor has not been analyzed in a placenta perfusion cotyledon model previously.

Our hypothesis was that PDE5 inhibitors will cross from the maternal to the fetal circuit and result in improved feto-placental arterial perfusion. The primary objective was to determine whether treatment in the intervillous space with sildenafil citrate or tadalafil attenuates the vasoconstrictive effect of U46619 in feto-placental arteries in an ex vivo dual cotyledon, dual perfusion human placental model. Secondary objectives included comparing the efficacy of sildenafil citrate versus tadalafil in this model and determining what PDE enzymes affected by these medicines are present in the normal term human placenta.

Methods

This protocol was approved through the Madigan Army Medical Center Investigational Review Board. Study data and materials will be made available on reasonable request to the corresponding author.

Placenta Perfusion Studies

The placenta perfusion technique was modified from the original model created by Glance et al.[19,20] Inclusion criteria were placentas from normal term pregnancies having unlabored scheduled cesarean deliveries. Exclusion criteria included active labor, rupture of membranes, fetal anomalies, preeclampsia, hypertension, fetal growth restriction, placental anomalies, abruption, chorioamnionitis, or placentas not suitable for the study because of tears or infarcts.

Immediately after delivery, placentas in the study were collected and transported to the placenta perfusion laboratory. The placentas were delivered to the laboratory within 5 minutes of delivery and examined for lacerations or infarcts. Then, the chorionic surface was inspected for a chorionic artery and vein pair supplying a cotyledon. These vessels were then cannulated with a 22-gauge intravenous catheter, and the baseline perfusate was infused into the fetal artery at a rate of 4 mL/min. After venous return was established from the corresponding fetal vein, a circular section of the placenta, ≈8 cm in diameter, including the selected cotyledon, was then excised. The cotyledon circuit was then placed in a temperature-controlled chamber maintained at 37°C. Two 21-gauge butterfly needles were then inserted through the chorionic surface into the intervillous space. Perfusion of the intervillous space was then established at 10 mL/min. In a similar manner, a second cotyledon from the same placenta was prepared. Perfusion of both cotyledons was established within 30 minutes after the placenta was delivered. The baseline solution was infused for 30 minutes into both the control and treatment cotyledons, establishing baseline perfusion.

The baseline perfusate for the maternal and fetal circuits consisted of a Hanks' balanced salt solution (Sigma Chemical Co, St. Louis, MO) mixed with gentamicin 5 mg/mL. Sterile technique was used throughout. Bovine albumin 2.0 g/L (Sigma) and sodium heparin 2000 U/L (Elkins-Sinn Inc, Cherry Hill, NJ) were added to the perfusate. Throughout the experiment, a 95% oxygen/5% carbon dioxide mixture was bubbled into the perfusate, and the pH was maintained between 7.35 and 7.45. The concentration of oxygen is reliable for placenta perfusion experiments in the absence of erythrocytes.[25,26] Standard commercial intravenous tubing was utilized for perfusion circuits. The intervillous perfusion rate was controlled by IMED (IMED Corp, San Diego, CA) pumps or the equivalent. The fetal perfusion rate was controlled by a Corpak 300D Enteral (Corpak Inc, Wheeling, IL) roller-type pump or the equivalent. For each cotyledon, an in-line transducer connected to a fetal monitor (Corometrics, Wallingford, CT) was used to measure the feto-placental arterial pressure.

Both cotyledons were preconstricted with a continuous infusion of U46619 (Cayman Chemical Co, Ann Arbor, MI), a thromboxane mimetic. This was done by infusing U46619 at a dose of 2 nmol/L into the maternal intervillous space and into the fetal chorionic artery of both the treatment and control cotyledons. The feto-placental arterial pressures were recorded over a 30 to 60-minute timeframe. This was done such that the baseline perfusion pressure (typically around 15–30 mm Hg) doubled or tripled in pressure (typically to about 40–60 mm Hg). After the preconstricted pressures were established, U46619 was continuously infused at 2 nmol/L for the remainder of the experiment into the maternal and fetal circuits for both cotyledons.

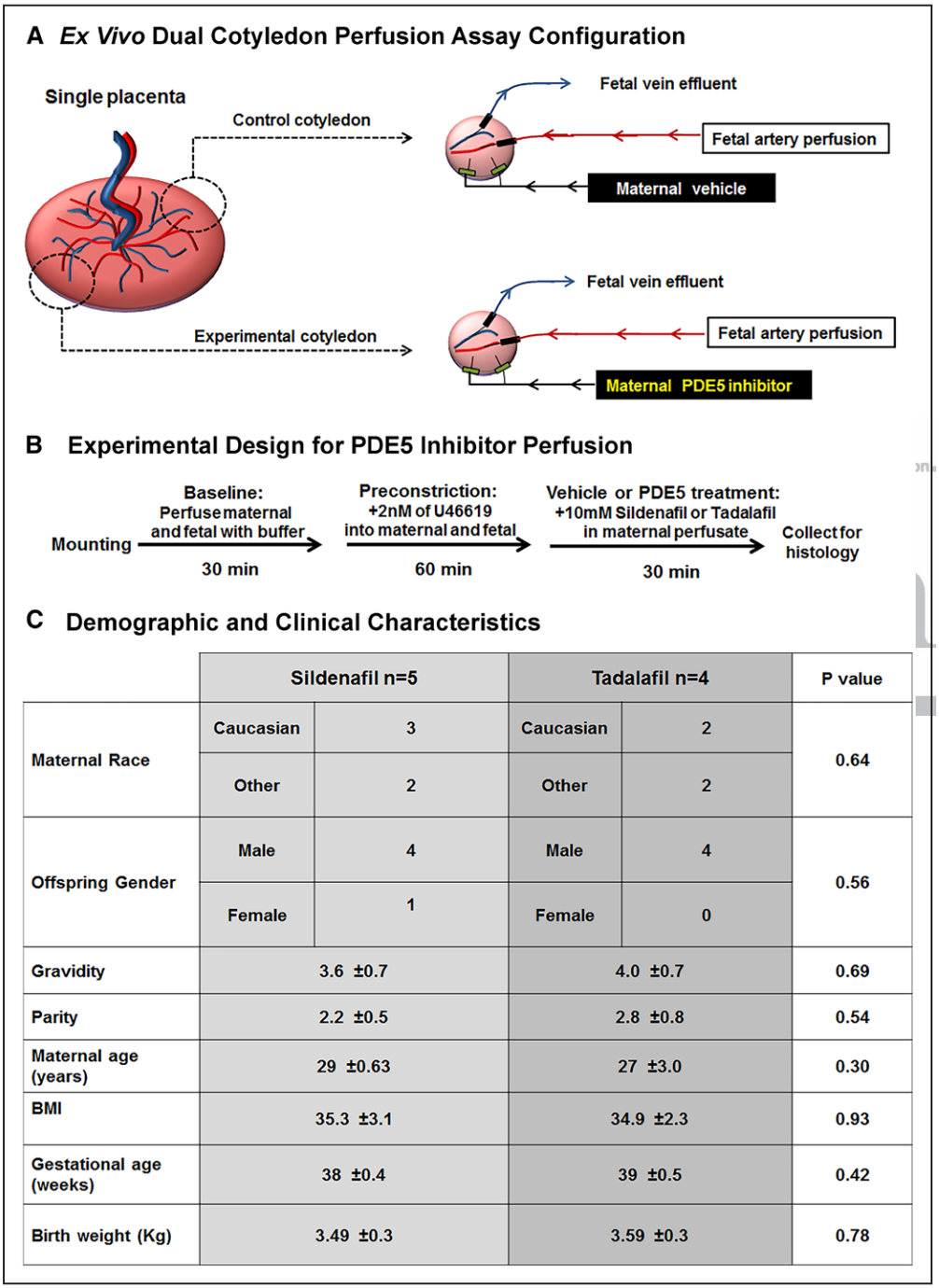
A random number table was used to determine which cotyledon would be the treatment specimen and which would be the control. The maternal intervillous space of the treatment cotyledon was switched over to a solution containing PDE5 inhibitor. We used sildenafil citrate

(Selleck Chemicals, Houston, TX), at a dose of 10 µmol/L for the first 5 placentas. This dose corresponds to the maximum concentration achieved in human pharmacokinetic trials.[18,27] We then used tadalafil (Selleck Chemicals, Houston, TX), at the same molar concentration of 10 µmol/L for the final 4 placentas. Feto-placental arterial pressures were recorded for 30 minutes, at which point tissue biopsies were collected for molecular/histological analyses.[25] Figure 1A and 1B for a summary of the experimental design.

A power analysis was done from our experience with previous placenta perfusion studies, we chose a 35% pressure drop as a significant effect.[23] To capture the same difference with the same SDs at an α level of 0.05 and with statistical power at 80%, a derived minimum sample size of 3 per placentas was determined to be sufficient. However, we increased our sample size to 5 per PDE5 inhibitor group. Statistical analysis was performed using a Student *t* test and ANOVA when appropriate.

Histology and Molecular Analyses

Assays were conducted following manufacturer protocols and standard practices; specific details are outlined in the Methods



C Demographic and Clinical Characteristics

	Sildenafil n=5		Tadalafil n=4		P value
Maternal Race	Caucasian	3	Caucasian	2	0.64
	Other	2	Other	2	
Offspring Gender	Male	4	Male	4	0.56
	Female	1	Female	0	
Gravidity	3.6 ±0.7		4.0 ±0.7		0.69
Parity	2.2 ±0.5		2.8 ±0.8		0.54
Maternal age (years)	29 ±0.63		27 ±3.0		0.30
BMI	35.3 ±3.1		34.9 ±2.3		0.93
Gestational age (weeks)	38 ±0.4		39 ±0.5		0.42
Birth weight (Kg)	3.49 ±0.3		3.59 ±0.3		0.78

Figure 1. Placenta perfusion experimental design and clinical characteristics. **A**, Diagram outlining the dual cotyledon, dual perfusion model where 2 cotyledons from a single placenta are simultaneously perfused through the maternal and fetal circuits. **B**, The experimental design followed in perfusion of PDE5 (phosphodiesterase type 5) inhibitors or the vehicle into the maternal circuit of preconstricted cotyledons. **C**, Maternal and fetal characteristics related to the placentas used in our study. All values represent the mean and ±errors represent the SEM. *P* values for the categorical data represented in rows 1 to 2 were compared by Fisher exact test. *P* values for rows 3 to 9 were generated by Student *t* test. BMI indicates body mass index.

in the online-only Data Supplement. Primers were generated by PrimerBank (Harvard) and are listed in Table S1 in the online-only Data Supplement.[28] Quantification of arterial diameters was generally followed from Buga et al.[29]

Results

All samples were from a scheduled cesarean, term placentas, without hypertension, diabetes mellitus, or other significant medical comorbidities. Demographic information was not significantly different between placentas used for sildenafil citrate and tadalafil treatments (Figure 1C).

Sildenafil Citrate Perfusion

Five placentas were chosen for this part of the experiment. First, a Hanks' basic salt solution was perfused into the maternal and fetal circuits for 30 minutes, establishing a baseline feto-placental arterial pressure of 20.8±1.4 mm Hg for the control cotyledons and 23.2±2.7 mm Hg treatment cotyledons (P=0.46). Then, the thromboxane mimetic, U46619, was continuously perfused into the maternal and fetal circuits for up to 60 minutes, increasing the feto-placental arterial pressure to an average of 48.4±1.7 mm Hg for the control cotyledons versus 51.0±3.6 mm Hg for the treatment cotyledons (P=0.24). This successfully established the preconstricted circuits for both cotyledons.

Next, sildenafil citrate was infused a continuous rate into the intervillous space of the treatment cotyledon for 30 minutes. At the same time, U46619 was infused at the same rate in the maternal and fetal circuits in both cotyledons. A significant reduction in the feto-placental arterial pressure in the sildenafil citrate cotyledon was reached by 15 minutes. The maximal improvement in perfusion pressure for the treatment group was at 25 minutes, which consisted of a decrease in feto-placental arterial pressure from 51 to 30 mm Hg (P=0.0297, 1-sided t test). At this time, the feto-placental arterial pressure decreased by 42% in the treatment group compared with 10.3% in the control group (Figure 2A).

Tadalafil Perfusion

Four placentas were selected with the above criteria. The methodology was identical for the sildenafil citrate experiments, except that the treatment cotyledon received tadalafil. The baseline perfusion pressures and preconstricted pressures

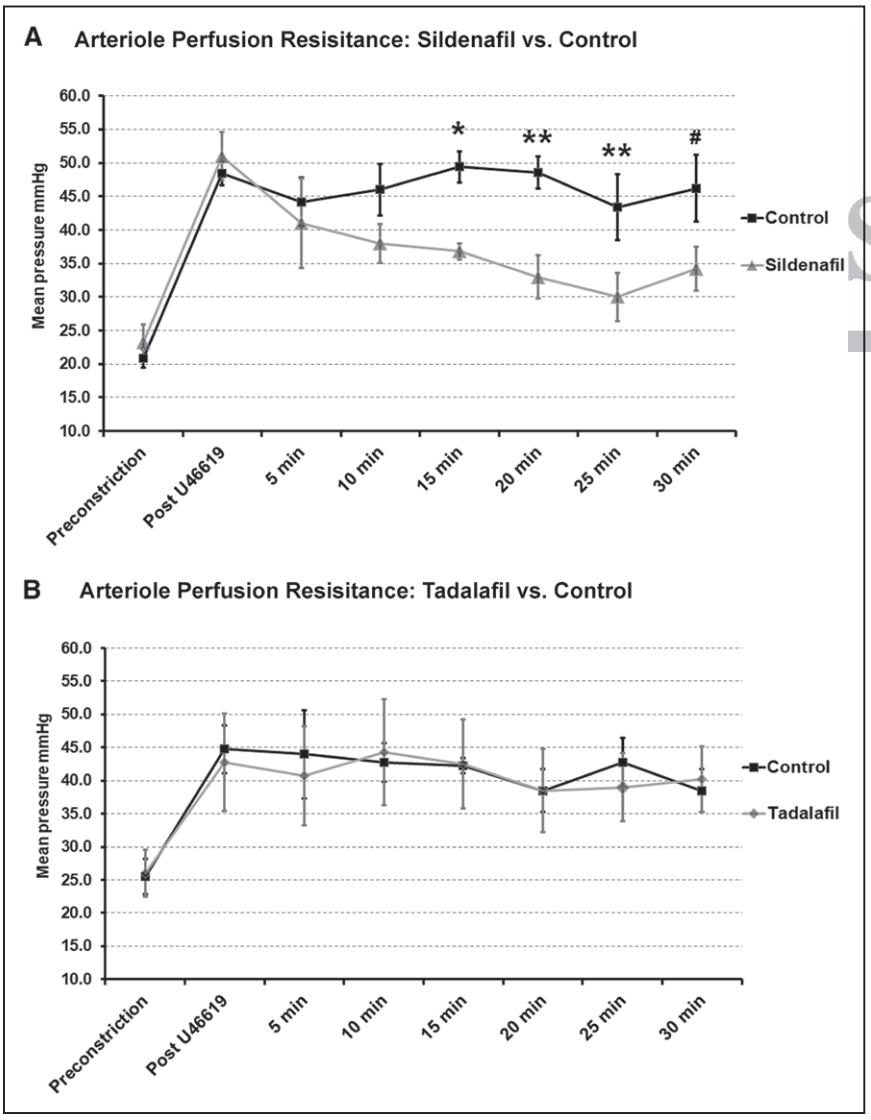


Figure 2. Treatment with sildenafil but not tadalafil decreases perfusion pressure in preconstricted feto-placental arteries. **A**, Placental arterial vascular resistance was recorded at the specified time points represented in the x axis. After 60 min of infusion with U46619, arterial pressures dramatically increased from the preconstricted baseline. At this point, continuous infusion of sildenafil through the maternal circuit of experimental cotyledons resulted in a significant decline of arterial pressure vs vehicle controls. **B**, After the same experimental design, perfusion of tadalafil did not reduce pressures in experimental vs control cotyledons. #P=0.05, *P<0.05, **P<0.005 by Student t test between experimental vs control cotyledons at each respective time point. Error bars represent the SEM.

were comparable to the sildenafil citrate placentas. With the infusion of tadalafil, there was no significant difference in feto-placental arterial pressures at any of the time points (Figure 2B).

Histology and Molecular Analyses

We collected biopsies from the placentas in our study before and after perfusion for histological and molecular analyses. In Figure 3A, hematoxylin and eosin staining of the demonstrates

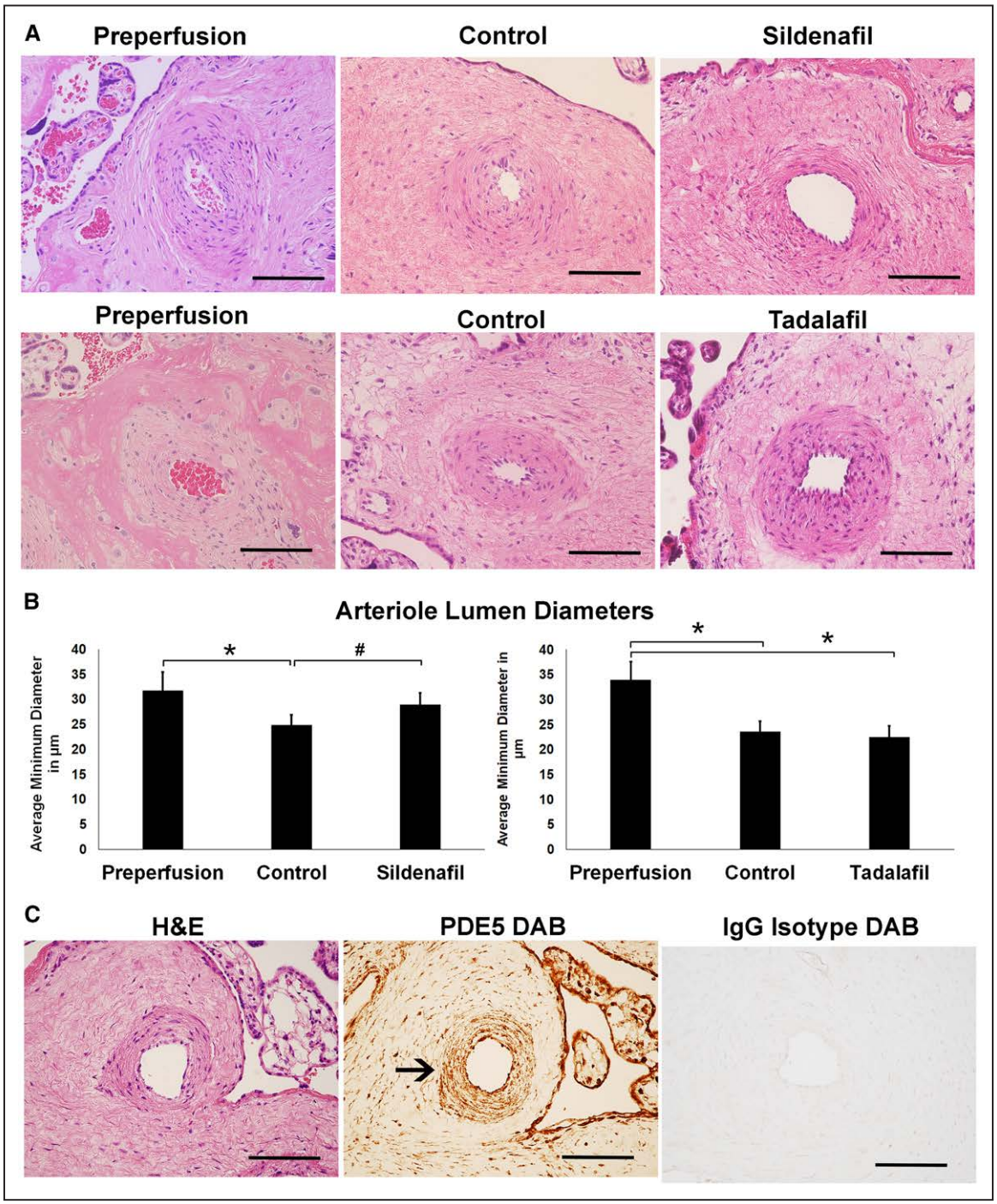


Figure 3. Histological analysis of feto-placental arteries corresponds with pressures and confirms PDE5 (phosphodiesterase type 5) in the vascular smooth muscle. **A**, Hematoxylin and eosin (H&E) stained cross-sections of placenta samples collected and fixed before perfusion (preperfusion) and after perfusion with the vehicle control or PDE5 inhibitors. Note, erythrocytes are abundant in the maternal and fetal compartments of only preperfused samples, indicating that the results observed with tadalafil were not attributed to clotting or insufficient perfusion. Scale bars=50 μm. **B**, The minimum diameters of the feto-placental arteries quantified from samples represented in Figure 2. Diameters in control vs sildenafil perfused samples were lower but not significant. Control and tadalafil samples were similar and significantly lower than preperfused samples from the same placentas. #P=0.09, *P<0.05 by Student t test. Error bars represent the SEM. **C**, H&E of a placenta sample perfused with sildenafil (**left**) and PDE5 staining with DAB (3,3'-diaminobenzidine) (**right**) on a serial section of the same vessel, highlights the presence of PDE5 in the vascular smooth muscle (arrow). An IgG isotype compared in parallel to the PDE5 staining shows an absence of nonspecific binding within the same placenta tissue. Scale bars=50 μm.

the thin endothelial layer surrounded by layers of smooth muscle in feto-placental arteries before and after perfusion with control or PDE inhibitor for sildenafil citrate and tadalafil, respectively. With sildenafil, arteries appeared more dilated. In contrast, arteries appeared similar to controls in cotyledons perfused with Tadalafil (Figure 3B). Figure 3C represents the quantification of arteriole diameters that support the pressure differences observed with sildenafil but not tadalafil. In Figure 3D, postperfusion histology was done on the placentas perfused with sildenafil citrate. On the left, hematoxylin and eosin staining shows fetal arteriole dilation. In the middle, antibody against PDE5 was used to locate where PDE5 is expressed, in this case in the smooth muscle and endothelium. On the right, an IgG isotype compared in parallel to the PDE5 staining shows an absence of nonspecific binding within the same placenta tissue.

Expression analysis for phosphodiesterase enzymes in samples collected preperfused from the same normal term human placentas was also performed. Figure 4A shows the levels of PDE enzymes. Importantly, *PDE5* and *PDE10* transcripts were both abundantly expressed. The expression level for PDE5 was significantly greater than PDE10. To confirm the translation and abundance of PDE10 Western blot analysis was conducted. Being that PDE10 can also bind sildenafil, we also compared perfused samples in this experiment. Figure 4B represents the abundance of PDE5A and 10A proteins in separate blots from the same set of samples. Once more PDE10 was significantly lower than PDE5 and there was no change in its abundance with perfusion. Though not significant ($P>0.05$), the abundance of PDE5 appeared lower with exposure to sildenafil citrate versus preperfused samples collected from the same placentas.

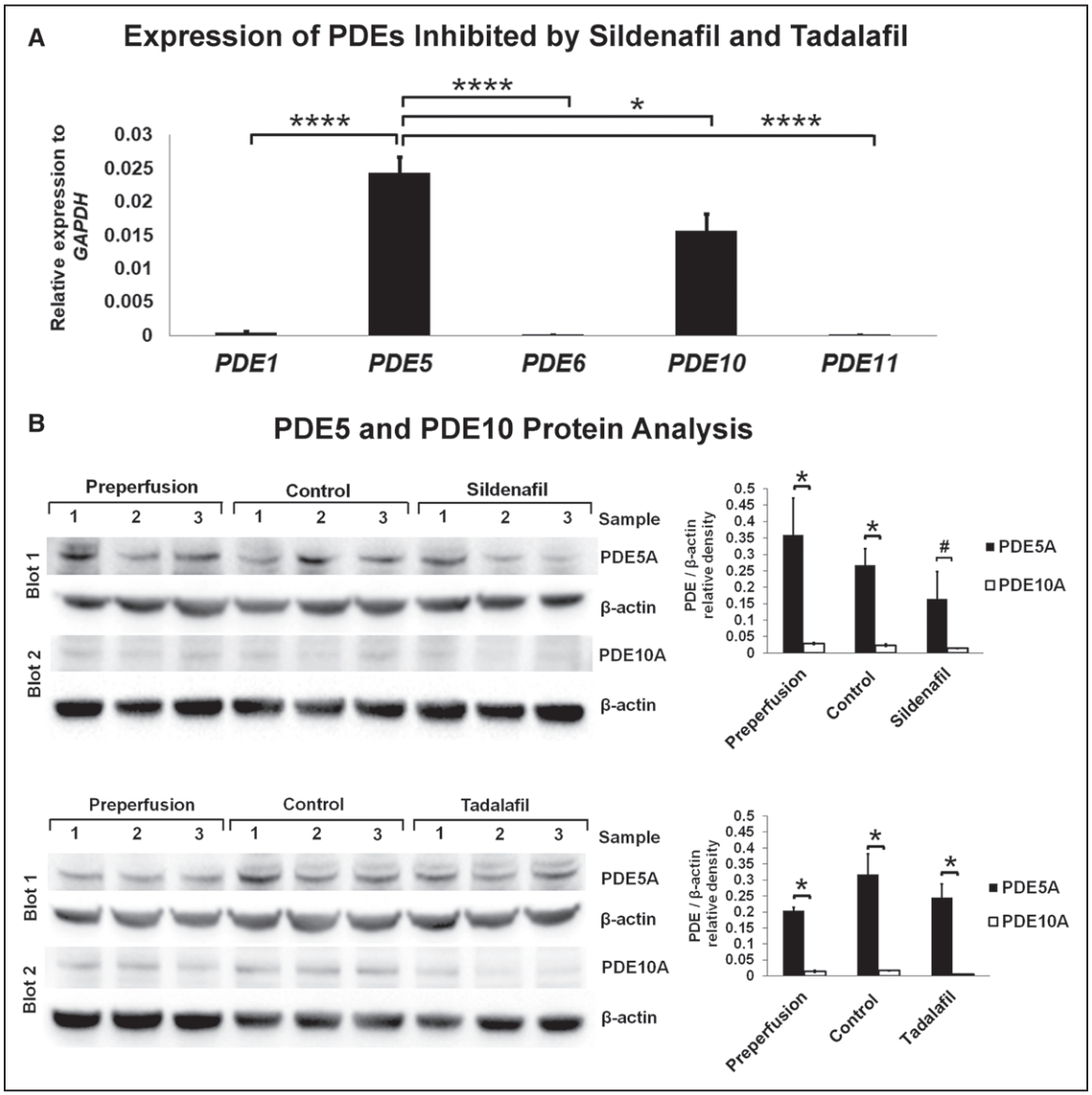


Figure 4. PDE5 (phosphodiesterase type 5) and 10 are differentially expressed in human placentas. **A**, The relative expression of PDEs inhibited by sildenafil and tadalafil analyzed in preperfused placenta biopsies (N=4 placentas). Only *PDE5* and *10* transcripts were present at significant levels. **P*<0.05, *****P*<0.00005 by Student *t* test. *P* value by ANOVA <0.005. Error bars=SEM. **B**, Western blot analysis confirms the presence of PDE5A and 10A proteins relative to β-actin in preperfused and experimental samples (N=3 per condition). From the same samples, separate membranes were run and probed for PDE5A (blot 1) and 10A (blot 2). #*P*=0.07, **P*<0.05, ***P*<0.005 by Student *t* test.

Discussion

We demonstrated that sildenafil citrate injected into the intervillous space significantly attenuated the vasoconstrictive effect of U46619 in feto-placental arteries in an ex vivo dual cotyledon, dual perfusion human placental model. We showed that sildenafil citrate improves fetal artery flow by 40% in our model. We further demonstrated that tadalafil injected into the intervillous space had no effect on attenuating the preconstriction in feto-placental arteries. We also demonstrated that the PDE5 enzyme is indeed present in the smooth muscle and endothelium of the normal term human placenta. Finally, we showed that among the 6 different PDE enzymes that sildenafil citrate and tadalafil target, only *PDE5* and *PDE10* were abundantly expressed in the human placenta. The translation of PDE10 protein was confirmed in comparison to PDE5. The fold difference between proteins was even greater suggesting that PDE5 is the predominant phosphodiesterase in the human placenta. When considering abundance and selectivity of sildenafil citrate, it is plausible that reversal of constriction in our perfusion experiments resulted from the inhibition of PDE5 and less likely from PDE10. These findings suggest that the PDE5 inhibitors could be used to affect the vascular tone and improve blood flow in the human placenta. This may support a human trial involving sildenafil citrate in the early growth-restricted fetus that is ongoing.[2]

Unexpectedly, tadalafil did not have any significant effect on the feto-placental arterial pressure in our model. Tadalafil, a more specific PDE5 inhibitor, is also known to be more potent than sildenafil citrate. Given its superior characteristics, we had anticipated a greater response with tadalafil when compared with an equal dose of sildenafil. In another experiment (not shown), tadalafil infused into the fetal artery did result in a reduction of the feto-placental arterial pressure of 26% (53 mm Hg after preconstriction, 39 mm Hg after tadalafil). Perhaps tadalafil does not cross the human placental barrier. Other possible explanations are that tadalafil is metabolized

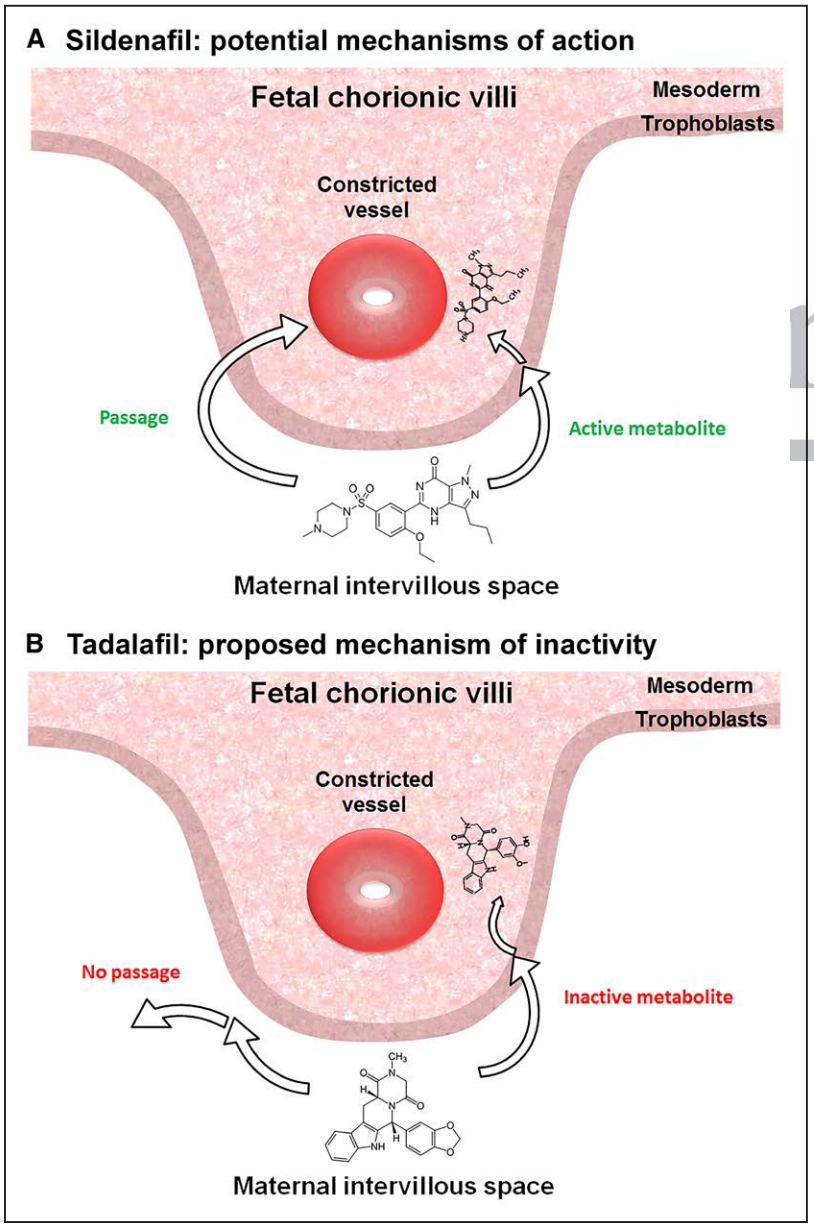


Figure 5. Potential mechanisms for placental PDE5 (phosphodiesterase type 5) selectivity. **A**, It is possible that sildenafil is transported or can permeate the maternal circuit to reduce placental vasoconstriction. A second possibility is that sildenafil is catabolized by placental trophoblasts and that its active metabolite reaches constricted vessels. **B**, In contrast to sildenafil, tadalafil may not be able to cross the placental barrier. In addition, tadalafil has no active metabolites and therefore would not work to reduce vasoconstriction unless it can reach the placental vasculature.

or actively transported from the fetal to the maternal side of the placenta. It is known that sildenafil citrate has an active metabolite, desmethyl sildenafil, which has a potency of 50% of sildenafil citrate and has similar selectivity for PDE5. In another study, Wareing et al[30] found that sildenafil citrate and desmethyl sildenafil were both present in umbilical cord samples, suggesting that this agent crosses the human placental barrier. Perhaps the response we detected in our study was because of the combination of sildenafil citrate and its active metabolite, and the fact that tadalafil has no active metabolite (summarized in Figure 5).[18]

There is limited data about tadalafil in pregnant women.[31,32] The evidence for sildenafil citrate usage in an animal in vivo fetal growth restriction studies has been inconsistent. Six studies of animal models of fetal growth restriction investigating the effects of sildenafil citrate on fetal growth or uterine blood flow were identified. Two mouse model studies and 1 sheep model showed an increase in neonatal growth with sildenafil citrate administration.[33–35] One study involving sheep showed an increase in uterine blood flow with sildenafil citrate administration.[36] In a rat model of fetal growth restriction, sildenafil citrate resulted in a higher number of stillbirths and reduced weight.[37] However, this study used a high dose of sildenafil citrate (50 mg/kg). In another sheep study, sildenafil citrate actually caused a decrease in uterine artery blood flow and weight loss.[38] However, in this study, the dosage of sildenafil citrate was higher than with the other sheep study (200 mg versus 150 mg). In the rat and latter sheep studies, it is possible that sildenafil citrate lowered the systemic blood pressure resulting in a steal of uterine perfusion to the other vascular beds.

Human in vitro studies of excised myometrial small arteries in growth-restricted pregnancies demonstrated increased endothelial-dependent vasodilation and decreased vasoconstriction with sildenafil citrate administration, suggesting a possible way to improve uteroplacental insufficiency.[39] Studies of the effectiveness of sildenafil citrate in improving the circulation of excised placental arteries have had mixed results—one study showed improvement and another showed no difference.[30,40] More recent results published from the STRIDER study involving sildenafil citrate for pregnancies complicated by severe early-onset fetal growth restriction, showed no difference in either neonatal morbidity or mortality or in delaying the interval to delivery.[17] This study, the largest to date, involved fetuses with an estimated fetal weight below the tenth percentile from 135 women enrolled at 19 different centers in the United Kingdom. Of note, the dosage of sildenafil citrate was 25 mg orally dosed 3× a day. This contrasted to the dose we used in our experiment, of 10 µmol/L. We derived this dosage from pharmacokinetic studies suggesting that the maximum effective dose of sildenafil citrate is achieved with an oral dose of 100 mg.[18] As with many drugs, the altered physiology of pregnancy can lower the bioavailable dosage.[41,42] Of note, drug levels were not reported in the STRIDER study. It is possible that a therapeutic dosage of sildenafil citrate was not achieved at the dose of 25 mg 3× daily. Therefore, it may be beneficial to repeat the STRIDER study using a higher dose of sildenafil citrate.

Our placenta perfusion model has many advantages over the previously studied human in vitro studies. The placentas continue to function in a milieu that is functionally and anatomically similar to placentas in actual pregnancies. Also, some of the previous animal studies have been done on placentas that are not hemochorial—by definition, the placentas in our model are hemochorial. Further, the continuous infusion of thromboxane mimetic is a model that simulates the phenotype of vasoconstriction-associated fetal growth restriction. Also, histology and molecular analyses can be done on the tissue after the perfusion experiments to evaluate PDE enzymes. Additionally, each run includes a treatment and control cotyledon, so each placenta serves as its own control. Finally, although not done in our study, one can use this model to study the transplacental passage of drugs.

The limitations of our study included the following: we did not evaluate growth-restricted placentas in our model. This is mainly because of the rarity of these placentas from planned cesarean sections. Further, placentas from growth-restricted pregnancies are technically difficult to catheterize. Next, this model simulates vasoconstrictive associated fetal growth restriction—importantly, there is no fetus that is being investigated. Our model simulates one common subtype of fetal growth restriction. However, fetal growth restriction has numerous causes that are not related to placenta blood flow. Finally, an experimental limitation of our study was that the infusion of PDE inhibitors was not conducted blinded.

Future research efforts could include pharmacokinetic and pharmacodynamic studies of PDE inhibitors in our model, to determine whether tadalafil crosses the human placenta barrier. Vardenafil, another PDE inhibitor that is more specific than sildenafil citrate, is more potent for the PDE5 enzyme, and also has an active metabolite, could also be studied using our model.

Perspectives

Mainly, this study supports the use of sildenafil over tadalafil for reversing constriction in human feto-placental arteries. The possibility that tadalafil may not cross the placental barrier or to a lesser degree than sildenafil deserves further evaluation. Currently, little is known about the mechanisms by which phosphodiesterases and other related molecules traffic into the human placenta. Understanding this process would enhance the development of more effective drugs for reversing feto-placental vasoconstriction. Second, we observed that PDE5 and PDE10 are the predominant phosphodiesterases with an affinity for sildenafil in placental tissue. PDE5 proved to be the most abundant in placentas from normal pregnancies. It remains possible that growth-restricted placentas express disproportionate amounts of PDE10 over PDE5. We can only speculate that dosing and interference from PDE10 influenced the results of the recent clinical trial. Altogether our findings suggest that higher doses of sildenafil may be necessary for improving placental blood flow in growth-restricted pregnancies. Finally, a more comprehensive evaluation of PDE5 inhibitors may reveal that vardenafil is more effective at reversing constriction of feto-placental arteries.

Acknowledgments

We acknowledge Amber D. Lane, Elisabeth M. Dornisch, and Jennifer R. Damicis for laboratory support. Dornisch, Damicis, and Lane were contracted employee of the Department of Clinical Investigation, with no conflicts or disclosures.

Sources of Funding

We are grateful for the Madigan Army Medical Center, Department of Clinical Investigation for funding this study and support by the Defense Health Agency, Research, Development, and Acquisition Directorate through the Clinical Research Intramural Initiative Program. The views expressed are those of the author(s) and do not reflect the official policy of the Department of the Army, Department of Navy, the Department of Defense, or the US Government.

Disclosures

We are employed by the US Military or by the Federal Government. The investigators have adhered to the policies for the protection of human subjects as prescribed in 45 CFR 46.

References

1. Bilardo CM, Wolf H, Stigter RH, Ville Y, Baez E, Visser GH, Hecher K. Relationship between monitoring parameters and perinatal outcome in severe, early intrauterine growth restriction. *Ultrasound Obstet Gynecol*. 2004;23:119–125. doi: 10.1002/uog.965.
2. Ganzevoort W, Alfirevic Z, von Dadelszen P, Kenny L, Papageorghiou A, van Wassenaer-Leemhuis A, Gluud 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*. 2014;3:23. doi: 10.1186/2046-4053-3-23.
3. Resnik R, Creasy RK. Intrauterine growth restriction. In: Creasy RK, Resnik R, Iams JD, Lockwood CJ, Moore TR, Greene MF, eds. *Creasy and Resnik's Maternal-Fetal Medicine: Principles and Practice*. 7th ed. Philadelphia, PA: Elsevier; 2014:743–755.
4. Berkley E, Chauhan SP, Abuhamad A; Society for Maternal-Fetal Medicine Publications Committee. Doppler assessment of the fetus with intrauterine growth restriction. *Am J Obstet Gynecol*. 2012;206:300–308. doi: 10.1016/j.ajog.2012.01.022.
5. Xu TT, Zhou F, Deng CY, Huang GQ, Li JK, Wang XD. Low-dose aspirin for preventing preeclampsia and its complications: a meta-analysis. *J Clin Hypertens (Greenwich)*. 2015;17:567–573. doi: 10.1111/jch.12541.
6. Roberge S, Nicolaides K, Demers S, Hyett J, Chaillet N, Bujold E. The role of aspirin dose on the prevention of preeclampsia and fetal growth restriction: systematic review and meta-analysis. *Am J Obstet Gynecol*. 2017;216:110 e116–120 e116.
7. Bujold E, Roberge S, Nicolaides KH. Low-dose aspirin for prevention of adverse outcomes related to abnormal placentation. *Prenat Diagn*. 2014;34:642–648. doi: 10.1002/pd.4403.
8. Boyce EG, Umland EM. Sildenafil citrate: a therapeutic update. *Clin Ther*. 2001;23:2–23.
9. Lacassie HJ, Germain AM, Valdés G, Fernández MS, Allamand F, López H. Management of eisenmenger syndrome in pregnancy with sildenafil and L-arginine. *Obstet Gynecol*. 2004;103(5 pt 2):1118–1120. doi: 10.1097/01.AOG.0000125148.82698.65.
10. Molelekwa V, Akhter P, McKenna P, Bowen M, Walsh K. Eisenmenger's syndrome in a 27 week pregnancy–management with bosentan and sildenafil. *Ir Med J*. 2005;98:87–88.
11. Streit M, Speich R, Fischler M, Ulrich S. Successful pregnancy in pulmonary arterial hypertension associated with systemic lupus erythematosus: a case report. *J Med Case Rep*. 2009;3:7255. doi: 10.4076/1752-1947-3-7255.
12. Baquero H, Soliz A, Neira F, Venegas ME, Sola A. Oral sildenafil in infants with persistent pulmonary hypertension of the newborn: a pilot randomized blinded study. *Pediatrics*. 2006;117:1077–1083. doi: 10.1542/peds.2005-0523.
13. Villanueva-García D, Mota-Rojas D, Hernández-González R, Sánchez-Aparicio P, Alonso-Spilsbury M, Trujillo-Ortega ME, Necoechea 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*. 2007;27:255–259. doi: 10.1080/01443610701194978.
14. Samangaya RA, Mires G, Shennan A, Skillern L, Howe D, McLeod A, Baker PN. A randomised, double-blinded, placebo-controlled study of the phosphodiesterase type 5 inhibitor sildenafil for the treatment of preeclampsia. *Hypertens Pregnancy*. 2009;28:369–382. doi: 10.3109/10641950802601278.
15. Lin TH, Su YN, Shih JC, Hsu HC, Lee CN. Resolution of high uterine artery pulsatility index and notching following sildenafil citrate treatment in a growth-restricted pregnancy. *Ultrasound Obstet Gynecol*. 2012;40:609–610. doi: 10.1002/uog.11142.
16. von Dadelszen P, Dwinnell 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. *BJOG*. 2011;118:624–628. doi: 10.1111/j.1471-0528.2010.02879.x.
17. Sharp A, Cornforth C, Jackson R, Harrold J, Turner MA, Kenny LC, Baker PN, Johnstone ED, Khalil A, von Dadelszen P, Papageorghiou AT, Alfirevic Z; on behalf of the STRIDER Group. Maternal sildenafil for severe fetal growth restriction (strider): a multicentre, randomised, placebo-controlled, double-blind trial. *The Lancet Child & Adolescent Health*. 2018;2:93–102. doi: 10.1016/S2352-4642(17)30173-6.
18. Mehrotra N, Gupta M, Kovar A, Meibohm B. The role of pharmacokinetics and pharmacodynamics in phosphodiesterase-5 inhibitor therapy. *Int J Impot Res*. 2007;19:253–264. doi: 10.1038/sj.ijir.3901522.
19. Glance DG, Elder MG, Myatt L. The actions of prostaglandins and their interactions with angiotensin II in the isolated perfused human placental cotyledon. *Br J Obstet Gynaecol*. 1986;93:488–494.
20. Glance DG, Elder MG, Myatt L. Prostaglandin production and stimulation by angiotensin II in the isolated perfused human placental cotyledon. *Am J Obstet Gynecol*. 1985;151:387–391.
21. Napolitano PG, Hoeldtke NJ, Moore KH, Calhoun BC, Christensen ED, Markenson GR, Hume RF, Jr. The fetoplacental pressor effects of low-dose acetylsalicylic acid and angiotensin II in the ex vivo cotyledon model. *Am J Obstet Gynecol*. 1997;177:1093–1096.
22. Hoeldtke NJ, Napolitano PG, Moore KH, Calhoun BC, Hume RF Jr. Fetoplacental vascular tone during fetal circuit acidosis and acidosis with hypoxia in the ex vivo perfused human placental cotyledon. *Am J Obstet Gynecol*. 1997;177:1088–1092.
23. Paonessa DJ, Shields AD, Howard BC, Gotkin JL, Deering SH, Hoeldtke NJ, Napolitano PG. 17-Hydroxyprogesterone caproate reverses induced vasoconstriction of the fetoplacental arteries by the thromboxane mimetic U46619. *Am J Obstet Gynecol*. 2006;195:1011–1014. doi: 10.1016/j.ajog.2006.06.041.
24. Gotkin JL, Celver J, McNutt P, Shields AD, Howard BC, Paonessa DJ, Napolitano PG. Progesterone reduces lipopolysaccharide induced interleukin-6 secretion in fetoplacental chorionic arteries, fractionated cord blood, and maternal mononuclear cells. *Am J Obstet Gynecol*. 2006;195:1015–1019. doi: 10.1016/j.ajog.2006.07.002.
25. Zelig CM, Paonessa DJ, Hoeldtke NJ, Hill DL, Foglia LM, Napolitano PG. Continuous infusion of 17-hydroxyprogesterone caproate into either the fetoplacental or intervillous circulation of a placental cotyledon attenuates vasoconstriction of the fetoplacental arteries by thromboxane mimetic U46619. *Am J Obstet Gynecol*. 2010;202:189.e1–189.e5. doi: 10.1016/j.ajog.2009.10.861.
26. Schneider H. Placental oxygen consumption. Part II: in vitro studies–a review. *Placenta*. 2000;21(suppl A):S38–S44.
27. 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*. 1999;29:297–310. doi: 10.1080/004982599238687.
28. Wang X, Spandidos A, Wang H, Seed B. PrimerBank: a PCR primer database for quantitative gene expression analysis, 2012 update. *Nucleic Acids Res*. 2012;40(Database issue):D1144–D1149. doi: 10.1093/nar/gkr1013.
29. Buga GM, Frank JS, Mottino GA, Hendizadeh M, Hakhamian A, Tillisch JH, Reddy ST, Navab M, Anantharamaiah GM, Ignarro LJ, Fogelman AM. D-4F decreases brain arteriole inflammation and improves cognitive performance in LDL receptor-null mice on a Western diet. *J Lipid Res*. 2006;47:2148–2160. doi: 10.1194/jlr.M600214-JLR200.
30. Wareing M, Myers JE, O'Hara M, Kenny LC, Taggart MJ, Skillern L, Machin I, Baker PN. Phosphodiesterase-5 inhibitors and omental and placental small artery function in normal pregnancy and pre-eclampsia. *Eur J Obstet Gynecol Reprod Biol*. 2006;127:41–49. doi: 10.1016/j.ejogrb.2004.06.014.
31. Kubo M, Tanaka H, Maki S, Nii M, Murabayashi N, Osato K, Kamimoto Y, Umekawa T, Kondo E, Ikeda T. Safety and dose-finding trial of tadalafil

administered for fetal growth restriction: a phase-1 clinical study. *J Obstet Gynaecol Res*. 2017;43:1159–1168. doi: 10.1111/jog.13345.
32. Sakamoto M, Osato K, Kubo M, Nii M, Tanaka H, Murabayashi N, Umekawa T, Kamimoto Y, Ikeda T. Early-onset fetal growth restriction treated with the long-acting phosphodiesterase-5 inhibitor tadalafil: a case report. *J Med Case Rep*. 2016;10:317. doi: 10.1186/s13256-016-1098-x.
33. 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*. 2013;8:e77748. doi: 10.1371/journal.pone.0077748.
34. 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*. 2010;140:251–258. doi: 10.3945/jn.109.114678.
35. Stanley JL, Andersson IJ, 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*. 2012;59:1021–1028. doi: 10.1161/HYPERTENSIONAHA.111.186270.
36. Zoma WD, Baker RS, Clark KE. Effects of combined use of sildenafil citrate (Viagra) and 17beta-estradiol on ovine coronary and uterine hemodynamics. *Am J Obstet Gynecol*. 2004;190:1291–1297. doi: 10.1016/j.ajog.2003.12.021.
37. Nassar AH, Masrouha KZ, Itani H, Nader KA, Usta IM. Effects of sildenafil in Nω-nitro-L-arginine methyl ester-induced intrauterine growth restriction in a rat model. *Am J Perinatol*. 2012;29:429–434. doi: 10.1055/s-0032-1304823.
38. 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*. 2009;200:102.e1–102.e7. doi: 10.1016/j.ajog.2008.08.029.
39. Wareing M, Myers JE, O'Hara M, Baker PN. Sildenafil citrate (Viagra) enhances vasodilatation in fetal growth restriction. *J Clin Endocrinol Metab*. 2005;90:2550–2555. doi: 10.1210/jc.2004-1831.
40. Maharaj CH, O'Toole D, Lynch T, Carney J, Jarman J, Higgins BD, Morrison JJ, Laffey JG. Effects and mechanisms of action of sildenafil citrate in human chorionic arteries. *Reprod Biol Endocrinol*. 2009;7:34. doi: 10.1186/1477-7827-7-34.
41. Ansari J, Carvalho B, Shafer SL, Flood P. Pharmacokinetics and pharmacodynamics of drugs commonly used in pregnancy and parturition. *Anesth Analg*. 2016;122:786–804. doi: 10.1213/ANE.0000000000001143.
42. Feghali M, Venkataramanan R, Caritis S. Pharmacokinetics of drugs in pregnancy. *Semin Perinatol*. 2015;39:512–519. doi: 10.1053/j.semperi.2015.08.003.

Novelty and Significance

What Is New?

- A comparison of the 2 common PDE5 (phosphodiesterase type 5) inhibitors, sildenafil and tadalafil, in a preconstricted human placental model that simulates growth restriction.

What Is Relevant?

- Sildenafil significantly reversed constriction versus controls. Surprisingly, tadalafil which is more potent than sildenafil did not reverse constriction.

Summary

Either tadalafil cannot cross to the placental arteries or is being degraded. In contrast, sildenafil can cross or is being degraded but its active metabolite reverses constriction.

Evaluation of Sildenafil and Tadalafil for Reversing Constriction of Fetal Arteries in a Human Placenta Perfusion Model

Robert B. Walton, Luckey C. Reed, Sarah M. Estrada, Stacey S. Schmiedecke, Diana L. Villazana-Kretzer, Peter G. Napolitano and Nicholas Ieronimakis

Hypertension. published online May 7, 2018;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0194-911X. Online ISSN: 1524-4563

SILDENAFIL CITRATE BUT NOT TADALAFIL REVERSES PRECONSTRICTION OF FETAL ARTERIES IN A HUMAN PLACENTAL PERFUSION MODEL.

Robert B. Walton[1], Luckey C. Reed[1], Sarah M. Estrada[1], Stacey S. Schmiedecke[1], Diana L. Villazana-Kretzer [1], Peter G. Napolitano[1], Nicholas Ieronimakis[2].

[1]Department of Obstetrics & Gynecology, Madigan Army Medical Center, Joint Base Lewis-McCord, Tacoma, WA, USA.
[2]Department of Clinical Investigation, Madigan Army Medical Center, Madigan Army Medical Center, Joint Base Lewis-McCord, Tacoma, WA, USA.

Supplementary Methods.

Sample Collection: Biopsies were collected from fresh cotyledons at collection and perfused cotyledons at the end of each experiment. Biopsies consisted of villous tissue cut approximately 1mm x 1mm towards the midline of each cotyledon, below the chorionic plate. One set of biopsies was collected for histology and immersed into formalin at five times the volume of tissue to fluid. Samples were fixed for 48 hours and then embedded in paraffin. A second set of biopsies was collected and flash frozen in liquid nitrogen and stored at -80^{o}C for RNA and protein analyses. These biopsies were later pulverized into powder and suspended in RNA and protein lysis buffer for the respective analysis.

Histology: Coronal cross-sections were cut 8um thick and cleared for H&E and DAB staining. For PDE5 staining, antigen retrieval was conducted with sodium citrate pH6.0 with 0.5% Tween20 for 30 minutes at 95^{o}C. Sections were then blocked overnight with PBS containing 1% BSA and 10% donkey serum. The next day polyclonal rabbit anti-PDE5 (Abcam, Cambridge, MA) was administered in 1% BSA overnight at 1:500**.** The staining was completed with ImmPRESS anti-Rabbit IgG kit followed by ImmPACT RTU DAB kit (both Vector labs,) in accordance with the manufactures' protocol. Fetal-placental arterial diameters were quantified using ImageJ by measuring the minimum diameter defined as the shortest internal diameter of the lumen. For these measurements ten arteries were randomly acquired at 40x and measured by individuals that were blinded to the treatments for each perfused and accompanying preperfused samples.

RNA Analysis: Total RNA was isolated using the RNeasy Lipid mini kit (Qiagen, Germantown, MD). First strand cDNA was generated using Applied Biosystems (Waltham, MA)) reverse transcriptase kit. Quantitative reverse transcription-PCR (qRT-PCR) was conducted with 30ng cDNA per reaction run in triplicate for each gene and sample using the FastStart SYBR green master mix from Roche (Pleasanton, CA). The

relative expression was calculated by ΔCt method by normalizing to *GAPD*. PDE primer sequences were.

Protein Analysis: Total proteins were isolated from samples lysed in RIPA buffer containing Halt protease inhibitor cocktail (both from Pierce, Waltham, MA) using standard procedures. Proteins were quantified using the Pierce BCA assay then denatured by boiling with BME at 95ºC for 10 minutes prior loading. 20ug of proteins was loaded and run per sample/well with 4-12% SDS gradient gels, then transferred onto cellulose membrane blocked with 5% BSA for 1 hour and probed with rabbit antibodies against PDE5A and PDE10A (both Abcam) at a dilution of 1:500 overnight. A secondary anti-rabbit HRP (Cell Signaling, Beverly, MA) at 1:1000 for 1 hour and proteins were visualized with chemiluminescence at the same exposure. Membranes were then stripped and probed with β-actin at 1:1000 for 1 hour (all from Cell Signaling). Due to similarities in their predicted size, separate blots were run for PDE5 and 10 in order avoid stripping of either protein. The relative density for each sample was quantified as PDE5 or 10 over β-actin using the gel analysis function in ImageJ.

Table S1. Primers used for qRT-PCR

Gene	Forward	Reverse	Produce Size (bp)
PDE1	**ATGGGGTCTAGTGCCACAGAG**	**GCACAGATGCCGCATATTCAAT**	**197**
PDE5	**GATCCTCGGTTCAATGCAGAA**	**ACAAAATGCCAAATAAGCAGCAA**	**187**
PDE6	**GACGTGTGGTCTGTGCTGAT**	**CTTGCCGTGGAGGATGTAGTC**	**189**
PDE10	**CCTGTGTATATTCACGCCACC**	**CCTCTTGGAAATCGTTCATCTCC**	**111**
PDE11	**TGATGACTTTTCTCTCGACGTTG**	**AAGCCACCTACACAGTGTCTC**	**159**
GAPDH	**GGAGCGAGATCCCTCCAAAAT**	**GGCTGTTGTCATACTTCTCATGG**	**115**