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Sildenafil crosses the placenta at therapeutic levels in a dually perfused human cotyledon model

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Abstract presented at the SMFM's 37th Annual Pregnancy Meeting, January 23rd-28th, 2017, Las Vegas, NV

Condensation:

Sildenafil crosses the term placenta *ex vivo* at a rate that is independent from the initial maternal concentration.

Short title:

Ex vivo passage of sildenafil across the human placenta

Implications and contributions:

A. *Why was this study conducted?* To investigate whether maternally administered sildenafil crosses the human placenta

B. *What are the key findings?* Sildenafil crosses the term placenta ex-vivo at a high rate, which is independent from the initial maternal concentration

C. *What does this study add to what is already known?* The results of this study suggest that, when sildenafil is administered to the mother at the currently used doses, clinically active levels of the drug are reached in the fetus

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Abstract

Background: Sildenafil is already administered during gestation in patients with pulmonary hypertension and is under evaluation as a treatment for several pregnancy complications, such as pre-eclampsia and intrauterine growth restriction. Animal studies have shown a potential therapeutic effect of the drug in fetuses with congenital diaphragmatic hernia, rescuing peripheral pulmonary vasculature and airway phenotype. When considering this drug for evaluation in a clinical trial, data on effective human placental drug passage are required.

Objective: We quantified transplacental passage of sildenafil in the *ex vivo* dually perfused cotyledon model.

Study Design: Six placentas were collected after term delivery from healthy volunteers, cannulated and dually perfused. Sildenafil citrate was added to the maternal circulation at two different concentrations: 500 ng/mL, representing the maximum tolerated concentration (N=3) and 50 ng/mL, representing the therapeutic concentration (N=3). Samples were collected from both the fetal and the maternal reservoir at 0, 6, 30, 60, 90, 120, 150 and 180 minutes, and the concentrations of sildenafil and its metabolite desmethyl-sildenafil were determined using high performance liquid chromatography. The fetal/maternal concentration ratio was calculated for each time-point. Transfer clearance was calculated as the rate of maternal to fetal passage/maternal concentration.

Results: Sildenafil crossed the placenta at both maximal and therapeutic concentrations. Maternal and fetal levels reached a plateau at 90-120 minutes. Transfer clearance was the highest during the first hour of perfusion, being 3.15 (range 2.14-3.19) mL/min for the maximum tolerated concentration and 3.07 (range 2.75-3.42) mL/min for the therapeutic concentration (NS). The feto-maternal concentration ratio significantly increased over time, up to 0.91 ± 0.16 for the maximal concentration and 0.95 ± 0.22 for the therapeutic

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concentration at the end of the perfusion (NS). Desmethyl-sildenafil was not detected in any sample.

Conclusion: Sildenafil crosses the term placenta at a relatively high rate *ex vivo*, suggesting that there is sufficient placental transfer to reach clinically active fetal drug levels at the currently used maternal doses.

Keywords: Congenital diaphragmatic hernia, Fetal therapy, Placenta perfusion model, Placental transfer, Sildenafil

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INTRODUCTION

Sildenafil is a selective phosphodiesterase 5 (PDE5) inhibitor approved by the Food and Drug Administration and European Medicines Agency for the treatment of erectile dysfunction and pulmonary hypertension in adults[1]. Over the past years, there has been a growing interest in the use of sildenafil during pregnancy for maternal and fetal conditions. We currently explore the efficacy of maternally administered sildenafil in the prevention of pulmonary hypertension in neonates with congenital diaphragmatic hernia (CDH). There is good evidence in two animal models (rat[2,3] and rabbit[4]) that antenatal transplacental administration of sildenafil rescues peripheral pulmonary vasculature and airway complexity in CDH fetuses. In most of the other conditions, sildenafil has been considered for its potential vasodilatory effect on the utero-placental vasculature. Even when given for maternal purposes, the potential fetal effects need to be considered, and therefore information on transplacental drug transfer is necessary to ensure fetal safety at specific maternal dose regimens. Fetal levels following maternal sildenafil administration have been measured in rats[2] and rabbits[4], showing a 50-80% ratio between fetal and maternal concentrations. However, there is a marked interspecies variability in placental transport and metabolism of xenobiotics[5,6]. Therefore, before clinical application human transplacental passage should be determined.

A harmless *ex vivo* testing method is to use the human placenta perfusion model. Dual perfusion of a single placental cotyledon is the only *ex vivo* experimental model to study human transfer of substances through intact placental tissue, and reliably predicts fetal exposure to maternally administered drugs[7,8]. In the present study, we aimed to determine maternal-fetal transplacental transfer rates of sildenafil using that model.

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MATERIALS AND METHODS:

Chemicals

Media for tissue preparation, washing and the experimental phase were prepared as previously described (summarized in Supplementary Table 1)[9]. Sildenafil solution and antipyrine (AP) were purchased from Merck (Overijse, Belgium).

Preliminary testing: drug degradation and adherence

Before the placental perfusion experiments, the influence of boundary conditions on sildenafil transfer was tested. Thermal- and photo-degradation and adherence of sildenafil to the perfusion system were assessed as described in the Supplementary Material.

Selection of donors and placenta collection

Fresh placentas from term (37-41 weeks gestation) uncomplicated pregnancies were collected immediately after delivery from consenting women at the University Hospital of Leuven, Belgium. Placentas from both vaginal births and cesarean sections were included. Exclusion criteria were factors potentially affecting placental integrity and transfer: chronic medication use during pregnancy, maternal diseases during pregnancy (including diabetes mellitus, hypertension, thyroid dysfunction), or any infection (chorioamnionitis and TORCH infections, or a positive anti–HIV, anti–HBV, or anti–HCV serology). This study was approved by the ethics commission of the University Hospital of Leuven (s54819).

Tissue preparation, washing and equilibration

Within 10 minutes after delivery, placental vessels were flushed with washing media, and the placentas were transported to the laboratory for the experimental procedures. We earlier described in detail the single cotyledon placenta perfusion system[9]. Briefly, the maternal side was inspected for tears and lesions and an intact cotyledon was selected. The supplying artery-vein pair on the fetal side was then cannulated (Flocare Pur sonde-MP Ch 6/60, Schiphol, The Netherlands). After confirmation of circulatory integrity, excess placental tissue was removed and the selected cotyledon was placed in a 37°C-controlled perfusion

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chamber. The intervillous space on the maternal side was perfused by five blunt catheters to establish the maternal circulation.

Double circulation was established with two peristaltic pumps with flows of 6 mL/min and 14 mL/min in the fetal and maternal circulation, respectively. The maternal and fetal compartments were then perfused with washing media for 20 minutes to remove any residual blood (*washing phase*). After the washing period, the perfusates of the maternal and fetal reservoir were changed to perfusion media, and the placenta was equilibrated for another 30 minutes to establish baseline parameters of pressure and cotyledon temperature and to reverse hypoxia (*equilibration phase*).

During all preparation phases, integrity of the circuit was tested by measuring the fetal outflow rate and the fetal and maternal pressure through a nanologger pressure measuring instrument (Gaeltec, Dunvegan, UK). The placental cotyledon circulation circuits that leaked or that did not achieve a stable pressure secondary to lack of vascular integrity were discarded.

Experimental phase

After the equilibration phase, both maternal and fetal perfusates were replaced with 250 mL fresh perfusion media, but this time 100 µg/mL AP (used as reference compound) and sildenafil at the selected concentration were added to the maternal medium. Two initial concentrations of sildenafil in maternal perfusate were tested: 500 ng/mL and 50 ng/mL, representing the maximum tolerated concentration (MC)[10] and the therapeutic concentration (TC) in humans[11]. Perfusion was maintained for at least 3 hours, over which time samples from maternal and fetal reservoirs were collected at time-points: 0, 10, 30, 60, 90, 120, 150, 180 min. Perfusate samples were centrifuged at 3000 rpm for 5 min at 4°C and stored in duplicates at -80°C. Concentration of AP was measured in one duplicate per time-point with high-performance liquid chromatography (HPLC) as previously described[9]. The other

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duplicate samples were analyzed for sildenafil and its main active metabolite desmethyl (DM)-sildenafil by HPLC with mass spectrometric detection at the VU Medisch Centrum Laboratory for Clinical Pharmacology and Pharmacy, Amsterdam, The Netherlands (Supplementary Material)[12].

At the end of the experiment, the perfused cotyledon was stored at -80°C and later sent for determination of sildenafil tissue concentration (BioNotus GCV, Niel, Belgium).

Quality control measures

pH, flow and glucose levels were measured in both compartments every 30 min to control the integrity and functionality of the perfusion. Pressure levels in the maternal and fetal compartment were measured continuously. After the experiment, the AP fetal/maternal concentration ratio (FM ratio) was measured to evaluate the overlap between maternal and fetal circulation. Only placentas meeting all the following quality control criteria were included in the analysis: maintenance of fetal pressure between 30 and 60 mmHg, stable pH of the perfusates, maintenance of a fetal outflow ≥ 95% of the fetal inflow, AP FM ratio ≥ 0.75 after 150 min of perfusion[13]. Moreover, only perfusions lasting at least 180 min were included.

Sample size and statistical analysis

Previous placenta perfusion studies have included three placentas evaluated for each condition to determine descriptive statistical parameters[14]. Therefore, we performed a preliminary analysis after three placental perfusions for both sildenafil concentrations (total n=6). Inter-experiment variability for the primary outcome measure (sildenafil FM ratio at steady state) was <30% in both groups. Hence, the sample size was not further increased.

The FM ratio of sildenafil was calculated for each time-point as follows[9]:

$$FM = \frac{[Sild\ Fet]t_x}{[Sild\ Mat]t_x}$$

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[Sild] is the concentration of sildenafil in the fetal (Fet) or maternal (Mat) compartment measured at a specific time-point.

To normalize data for inter-individual differences between placentas, the normalized FM ratio was calculated as a ratio between sildenafil and AP FM for each time-point[15]:

$$Normalized\ FM = \frac{Sildenafil\ FM\ ratio\ t_x}{AP\ FM\ ratio\ t_x}$$

In order to quantify the sildenafil transfer capacity from the maternal to the fetal compartment, the transfer clearance (Cl) was calculated as the rate of fetal excretion/maternal concentration[16]:

$$Cl = \frac{[Sild\ Fet]t_1 * Vfet\ t_1 - [Sild\ Fet]t_0 * Vfet\ t_0}{(t_1 - t_0) * [Sild\ Mat]_{mid}}$$

Herein, sildenafil concentrations are measured at the end (t_1), beginning (t_0) or at the midpoint ('mid') of the respective time period, and Vfet is the volume of the fetal compartment at the same time-point.

The transfer percentage (T%) was calculated in order to relate the amount of sildenafil measured in the fetal or maternal compartment to that initially present in the maternal circulation:

$$T(\%) = \frac{[Sild]t_x * V_x}{[Sild\ Mat]t_0 * V\ Mat_0} * 100$$

$[Sild]t_x$ is the concentration of sildenafil in the fetal or maternal compartment at the examined time-point, V_x is the volume of the fetal or maternal compartment at the same time-point, $[Mat]t_0$ is the concentration of sildenafil in the maternal compartment measured at the beginning of the perfusion and $VMat_0$ is the volume of the maternal compartment at the beginning of the perfusion.

Finally, the proportion of sildenafil detected in the cotyledon was calculated as:

$$Tissue\ Sild\ \% = \frac{[Tissue\ Sild] * \mathrm{cot}\, weight}{[Sild\ Mat]t_0 * V\ Mat_0}$$

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[Tissue Sild] is the average sildenafil concentration in six randomly selected pieces of the perfused cotyledon at the end of the experiment; cot weight is the weight of the cotyledon. All values are expressed as median (range). Mann-Whitney test was used to compare continuous variables, while Fisher's exact test was used for dichotomous variables. Differences between placentas perfused with sildenafil at MC and TC over time were determined by two-way ANOVA. A 2-tailed $p<0.05$ was considered significant. All comparisons were performed using Prism for Windows V.5.0 (GraphPad software, San Diego, California).

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RESULTS

Preliminary tests

Sildenafil concentration was not altered by heat and light exposure (Supplementary Figure 1A). However, when the maternal and fetal experimental circuits were perfused with sildenafil solution, sildenafil concentration decreased over time, reaching a plateau at 120 minutes. As thermal- and photo-degradation were already excluded by the previous experiment, such decrease in sildenafil concentration was attributed to adherence of the drug to the tubing. A non-linear binding curve best fitted the adherence data (Supplementary Figure 1B, R^2=0.96 for both the fetal and the maternal compartment). In the subsequent transfer experiment, the percentage of sildenafil not found in the maternal and fetal compartment was calculated by correcting sildenafil concentrations for adherence to tubing according to the equation described in Supplementary Methods.

Placental experiments

Out of 16 collected placentas, 12 (75%) were successfully cannulated and placed in the perfusion system. Pre-perfusion showed no leakage in 7 cases (44%), for which the experimental phase was initiated. One case did not meet the quality control criteria and was therefore excluded, resulting in 6 placentas (38%) that could be included in the analysis (n=3 for each sildenafil concentration tested). Table 1 shows the clinical and placental characteristics of the successfully perfused placentas. No differences were observed between placentas perfused with sildenafil at MC and TC.

For both initial maternal concentrations, sildenafil was detected in the fetal compartment and maternal and fetal levels reached a plateau at 150-180 minutes (Figure 1). Median sildenafil concentration at equilibrium (150 minutes) in the fetal compartment was 70 (67-86) ng/mL and 10 (7-10) ng/mL for initial maternal MC and TC respectively. DM-sildenafil was not detected in any sample.

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Transfer parameters are displayed in Table 2. For both MC and TC, the maximal placental clearance occurred during the first hour of perfusion (Cl 3.15 (2.14-3.19) and 3.07 (2.75-3.42) mL/min for MC and TC respectively, p=0.9). The FM ratio significantly increased over time (p<0.0001), up to 0.94 (0.74-1.05) and 0.90 (0.75-1.20) at the end of the perfusion (180 minutes) for MC and TC respectively. Two-way ANOVA analysis did not show any significant difference in FM ratio between the two initial maternal concentrations (Figure 2A and 2B). A similar temporal pattern was observed for the normalized FM ratio (Figure 2C). At equilibrium, the FM ratio for sildenafil was comparable to that of the control substance AP, leading to a normalized FM ratio of 0.86 (0.83-1.09) and 1.03 (0.76-1.08) for MC and TC respectively (p 0.9). Again, no difference was observed between the two groups (two-way ANOVA p 0.9).

After 150 minutes of perfusion, 14 (13-16)% and 13 (12-14)% of the compound initially added to the maternal circulation was detected in the fetal compartment for MC and TC respectively (p 0.5, Figure 3A). At the same time-point, only 16 (16-19)% and 18 (13-19)% of the initial sildenafil amount was detected in the maternal circuit for MC and TC (p 1, Figure 3B). Even when adherence of sildenafil to the tubing was taken into account (Supplementary Methods), this resulted in 40 (39-44)% and 42 (38-54)% of sildenafil not detectable in either the maternal or fetal compartment (Figure 3C). Of interest, the percentage of undetected sildenafil over time was significantly higher in placentas perfused with sildenafil at TC than MC (two-way ANOVA p=0.017, Figure 3C).

For all experiments, sildenafil was detected in placental tissue at the end of the perfusion. With the exception of one outlier in the TC group, sildenafil tissue concentrations were comparable (458 (379-517) vs 360 (61-463) for MC and TC respectively, p=0.4). Tissue Sild % was 9 (7-30) % for TC and 56 (17-62) % for MC (p=0.2).

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COMMENT

We demonstrated that sildenafil crosses the human term placenta *ex vivo* at a rate independent from the initial maternal concentration, when this is within the therapeutic range.

Sildenafil is increasingly being used during pregnancy for indications other than maternal pulmonary hypertension, such as preeclampsia[17], intrauterine growth restriction (IUGR)[18,19], oligohydramnios[20] and fetal distress during labor[21]. A systematic review demonstrated that sildenafil administration is safe for the mother[22]. We and others are now considering transplacental sildenafil administration to stimulate lung development in fetuses with CDH[2,4]. For all other indications, the major target of the drug is not the fetus but the placenta: through a selective vasodilatory effect, sildenafil may enhance utero-placental blood flow, hence improve fetal well-being. Up to now, studies have not focused on sildenafil transfer to the fetus. Our literature search did not reveal any publications on fetal sildenafil levels after maternal administration in humans. The only study reporting detectable umbilical cord blood sildenafil levels was done in pre-eclamptic women[17], a condition in which placental transfer may not be representative for other disorders[23]. Furthermore, this study did not report any quantitative data. However, recently there has been significant global interest following the suspension of the Dutch STRIDER trial on intrauterine growth restriction, “because of likely lack of benefit and of a potential signal of harm relating to an increased incidence of persistent pulmonary hypertension”[24]. Even though the association with pulmonary hypertension was not confirmed by other trials, this finding mandates additional studies on fetal safety. Transplacental transfer of sildenafil to the fetus after maternal administration represents therefore essential safety information, next to the specific effects of the drug on the target fetal organ(s).

Hutson et al. systematically reviewed studies comparing *ex vivo* maternal and fetal concentration simulations in perfused placentas to data obtained from women in labor and demonstrated for 70 different drugs that this model is highly representative yet precludes

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human experimentation[7]. That review also provided recommendations for standardization of placenta perfusion experiments, which we followed in our study.

In our experiment, the FM ratios of sildenafil and AP at equilibrium are likely to be indicative of the maternal steady state *in vivo*. These ratios, as well as sildenafil concentrations, remained relatively constant after 120 minutes (Figures 1 and 2), which suggests that sildenafil crosses the placenta following a concentration gradient. The relatively low molecular weight of sildenafil (474 Da) makes it likely that sildenafil crosses the placenta via passive diffusion, or with limited active transport. The moderately lipophilic character of sildenafil (LogP 1.38) makes it also plausible that it passes the placental barrier very effectively.

Despite the high FM ratio (more than 90% at the end of the perfusion), at equilibrium only 25% of the initial amount of sildenafil was detected in the fetal compartment, while more than 40% of the drug could not be detected in the maternal or fetal reservoir. Since the potential drug degradation and adherence were already taken into account, we hypothesized that part of the drug might accumulate in the placental tissue. Sildenafil has been shown to act on the human placenta, which is consistent with PDE5 being abundantly expressed in placental arteries[25]. Both *in vitro* contractility studies[26] and *in vivo* Doppler studies[27] have shown that sildenafil vasodilates the feto-placental circulation. We therefore measured the sildenafil tissue level in the cotyledons at the end of the perfusion. Cotyledonary sildenafil levels were comparable between the two dose groups. Although not statistically significant, there was a trend towards a higher proportion of the initial sildenafil amount detected in the cotyledon for TC (median 56%) vs MC (median 9%). Also, the proportion of sildenafil not demonstrable in the maternal and fetal compartments, was higher in placentas perfused with sildenafil at low concentrations. Taken together, these results might indicate that the binding sites in the placenta get saturated at high sildenafil concentrations, further supporting the hypothesis that

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the drug accumulates in the placenta. The variability of sildenafil tissue levels within groups might be explained by a variability in cotyledonary weights and potentially by technical issues in the storage or processing of one of the tissue samples (the outlier in the TC group).

Another possibility is that sildenafil undergoes biotransformation to a metabolite different from DM-sildenafil. We did not detect DM-sildenafil in the maternal or fetal compartment at any time-point, indicating that this metabolic pathway is not active in the term placenta, at least *ex vivo*. We did however *not* check for other metabolites because demethylation via CYP3A4 is the major metabolic pathway of the drug, and because DM-sildenafil is the only known metabolite with relevant pharmacological activity (50% of sildenafil activity)[28]. A minor metabolic route for sildenafil is through CYP2A4. However, studies on other drugs have shown that this isoform is involved in the liver but not placenta metabolism[29]. Nevertheless, the existence of different metabolic pathways through isoforms active in the placenta, such as CYP19 and CYP1A, cannot be excluded[29].

Some limitations of our study deserve discussion. We did not perform feto-maternal perfusion experiments. Therefore, the contribution by the placenta in clearing the drug from the fetal circulation has not been measured directly. Previous studies have shown that sildenafil is a substrate for P-glycoprotein[30]. P-glycoprotein has a unidirectional effect on transcellular transport; in the placenta, it transports substrates from the fetal to the maternal side. However, the FM ratio at equilibrium does not support a major impact of P-gp on fetal sildenafil distribution. Most likely, passive permeation rates of sildenafil are compensating largely for transporter-mediated processes.

Second, our *ex vivo* experiments were performed using term placentas yet not mid-gestational placentas. The permeability of the placental barrier and the transport of drugs increases throughout pregnancy. As a result, we cannot make deductions to earlier gestational ages, when placental structure is vastly different. The gestation-related changes in placental

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transporters and metabolism also need to be taken into account. Even though CYP3A4 mRNA has been detected in term placentas, its activity at this stage of gestation is partially inhibited[31], leading to a reduced placental metabolization of sildenafil at term. Our findings on DM-sildenafil confirm this hypothesis. However, this might not be the case at earlier gestational ages. Access to preterm placentas is difficult because those are sent for pathologic assessment for clinical diagnostic purposes. Even if available, the cause for preterm delivery (such as infection, preeclampsia or growth restriction) may affect the normal placental structure, transfer and metabolic activity, thereby making these placentas less representative of the *in vivo* situation.

Finally, the placenta perfusion model does not fully account for non-placental pharmacokinetic factors, such as maternal and fetal drug clearance, as well as distribution in the maternal and fetal compartments.

Despite these limitations, the placenta perfusion model is the best experimental model to study the potential of transplacental drug delivery, providing a bridge between translational studies and the clinical setting. Although animal studies can be very useful, results cannot always be clinically extrapolated because the placenta is a species-specific mammalian organ.

In conclusion, our study suggests that maternally administered sildenafil might be transferred to the fetus at a relatively high rate, even though a significant proportion of the drug is either retained in the placenta or metabolized. Given the complexity of the pharmacokinetics during pregnancy, further clinical studies are necessary to fully understand sildenafil transfer during gestation.

We will explore the pharmacokinetics of sildenafil and its maternal and fetal safety throughout the second half of gestation in a pregnancy–physiologically based pharmacokinetic modeling[32] study and a phase I clinical study (EudraCT: 2016-002619-17).

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Table 1: Clinical characteristics of patients and placentas studied. Results are expressed as median (range) for continuous variables, and as number (%) for dichotomous variables

	MC (N=3)	**TC (N=3)**	**p-value**
Gestational age, weeks	38.4 (37.2-39.6)	38.6 (38.5-40.3)	0.2
Vaginal delivery	2/3 (67%)	1/3 (33%)	0.5
Placental weight, g	800 (710-860)	720 (500-880)	0.6
Cotyledon weight, g	27.2 (24.7-77.0)	33.5 (24.4-42.8)	0.9
Cotyledon/placental weight (%)	3.8 (2.9-9.6)	4.9 (3.8-5.9)	0.9
Delivery to flushing time, min	6 (5-8)	7 (5-8)	1
Delivery to perfusion time, min	68 (48-81)	58 (50-59)	0.7

MC: maximum tolerated concentration; TC: therapeutic concentration; n: number; g: grams; min: minutes

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Table 2: Placental transfer parameters for the maximum tolerated (MC) and therapeutic (TC) sildenafil concentration. Results are expressed as median (range)

	MC	TC	p-value
Transfer clearance (0-60 min), mL/min	3.15 (2.14-3.19)	3.07 (2.75-3.42)	0.9
Transfer clearance (60-120 min), mL/min	1.26 (0.73-2.21)	1.36 (-0.72-2.71)	0.9
FM ratio at equilibrium (150 min)	0.93 (0.70-1.04)	0.83 (0.71-1.00)	0.9
Normalized FM ratio at equilibrium (150 min)	0.86 (0.83-1.09)	1.03 (0.76-1.08)	0.9
T% fetal at equilibrium (150 min)	14 (13-16)	13 (12-14)	0.5
T% maternal at equilibrium (150 min)	16 (16-19)	18 (13-19)	1
Tissue concentration at the end of the experiment (180 min), ng/g	458 (379-517)	360 (61-463)	0.4
Tissue Sild %	9 (7-30)	56 (17-62)	0.2

Min: minutes; FM ratio: feto-maternal ratio; T%: percentage of transfer

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Figure legends

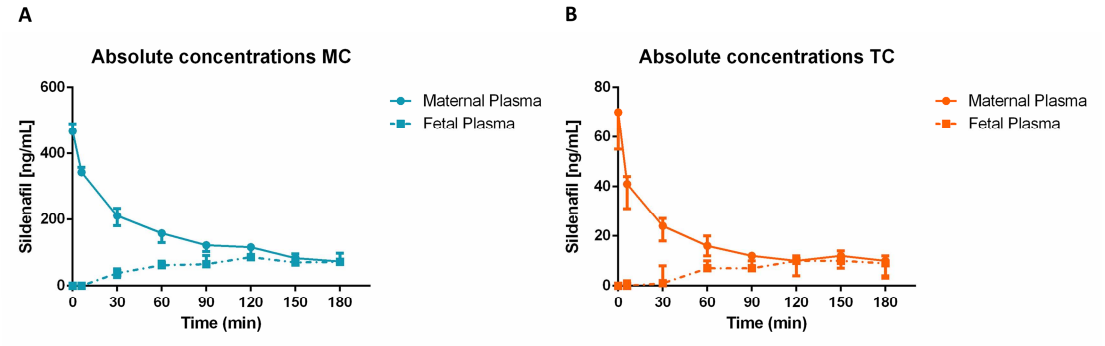
Figure 1: Concentration-time profile of sildenafil in the maternal and fetal compartments over the 3 hour-perfusions for the MC group (A) and the TC group (B). Points represent medians, error bars represent ranges.

Figure 2: Feto-maternal (FM) ratios over time. A: FM ratios are shown for individual placentas. FM ratios (B) and normalized FM ratios (C) are grouped according to the initial maternal concentration. Points represent medians, error bars represent ranges.

Figure 3: Percentage of the initial sildenafil amount detected in the fetal (A) or maternal (B) compartment or undetected (C). The percentage of sildenafil not found in the maternal and fetal compartment (C) was calculated by correcting sildenafil concentrations for adherence to tubing according to the equation described in Supplementary Methods. The p value refers to differences between placentas perfused with sildenafil at therapeutic concentration (TC) and maximum tolerated concentration (MC). Points represent medians, error bars represent ranges.

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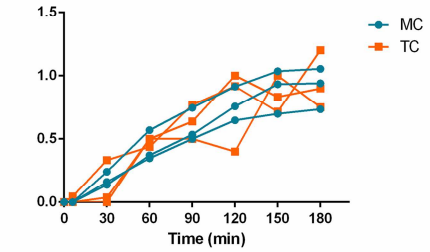
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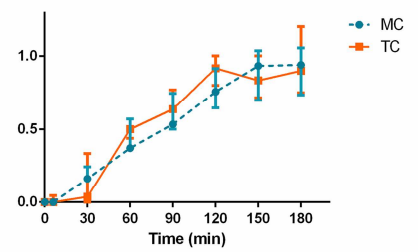
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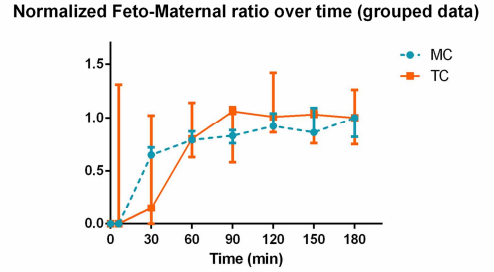
A



B



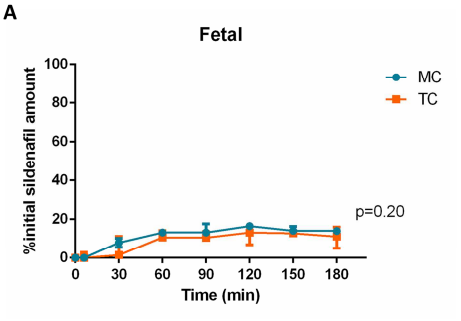
C



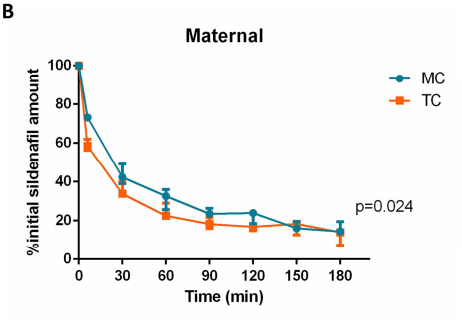
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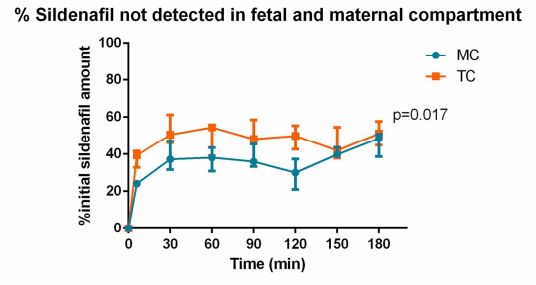
A



B



C



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Supplementary table 1: Composition of the different media used during the perfusion experiments

Medium use	Composition
Flushing medium upon receiving	Krebs buffer; Glucose (2.61 g/L); Heparin (25,000 UI/L)
Maternal washing medium	Krebs buffer; Glucose (2.61 g/L); Heparin (25,000 UI/L) Bubbled with 95% O2 and 5% CO2
Fetal washing medium	Krebs buffer; Glucose (2.61 g/L); Heparin (25,000 UI/L) Bubbled with 95% N2 and 5% CO2
Maternal equilibration and experimental medium	EBSS, Glucose (1.6 g/L), Heparin (25,000 UI/L), L-glutamine (1%), Penicillin- streptomycin (1%), BSA (30 mg/mL) Bubbled with 95% O2 and 5% CO2
Fetal equilibration and experimental medium	EBSS Glucose (1.6 g/L), Heparin (25,000 UI/L), L-glutamine (1%), Penicillin- streptomycin (1%), BSA (40 mg/mL) Bubbled with 95% N_2 and 5% CO_2

Legend: EBSS= Earle's balanced salt solution; BSA= bovine serum albumin.

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Supplementary methods:

Preliminary testing: drug degradation and adherence

In order to evaluate thermal- and photo-degradation, sildenafil was added to the perfusion medium at 500 ng/mL. The solution was kept at a temperature of 37°C, with (for photodegradation) or without exposure to light, and samples were taken at time 0, 60, 120 and 180 minutes. Sildenafil concentration was determined at each time-point. Adherence of sildenafil to the perfusion system was assessed by reproducing the experimental setting without the human cotyledon in the chain. Perfusion medium containing sildenafil at a concentration of 250ng/mL was added to two closed circuits representing the maternal and fetal reservoir. Both the fetal and maternal reservoir were sampled at 0, 60, 120 and 180 minutes from the beginning of perfusion. After washing and perfusion with equilibration medium, the experiment was repeated. All these tests were performed in duplicate.

In the transfer experiment, absolute sildenafil concentrations over time in both the maternal and fetal compartment were normalized by the degree of adherence to tubing using the following equation:

$$[Sild\ nor]at\ t(x) = \frac{[Sild\ meas]at\ t(x) * 100}{100 - \%adherence\ at\ t(x)}$$

[Sild nor] is the adjusted sildenafil concentration at the examined time-point, [Sild meas] is the measured sildenafil concentration and %adherence is the adherence rate at the same time-point.

Details on sildenafil and DM-sildenafil assay on perfusates

Concentration of sildenafil and DM-sildenafil on perfusates was measured using liquid chromatography-mass spectrometry at the Department of Clinical Pharmacology and Pharmacy, VU University Medical Centre, Amsterdam, The Netherlands. The assay is linear over 1-600 ng/mL for sildenafil and 1-300 ng/mL for DM-sildenafil, with the lower limits representing the lower limits of quantification (LLOQ) of sildenafil and its metabolite. Its

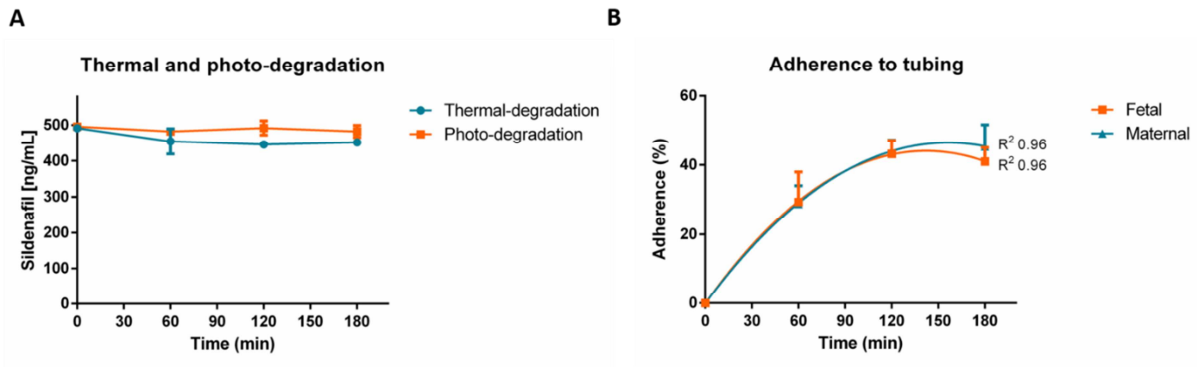
intra- and inter-assay accuracies range from 92.8-103.2% and intra- and inter-assay imprecision does not exceed 15%.

Determination of sildenafil concentration in placenta tissue

For each cotyledon, six randomly selected pieces weighing 300 mg each were selected for analysis. Tissue preparation was as previously described [1]

To each piece, 100 µL internal standard (200 ng/mL loperamide) and 900 µL water or standard sildenafil dilutions in water was added. After homogenization, the samples were centrifuged for 10 min at 4000 rpm at 4 °C. After homogenizing the samples and centrifuging for 5 min at 4000 rpm at 4 °C, To 500 µL supernatants 50 µL perchloric acid (35%) was added. The samples were vortexed twice for 30 sec and centrifuged for 10 min at 16000 rpm at 4 °C. The clear supernatants were pipetted into micro vials for automatic injection. Injection volume was 1 µL. Sildenafil concentration was determined with the assay described above. The sildenafil mean concentration for each placenta (n=6 pieces) was then calculated.

Supplementary Figure 1: Results of the preliminary tests. A: sildenafil concentration over time during heat and light exposure; B: fit curve for drug adherence to tubing



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