MRI Characterization of Blood Flow and Oxygen Delivery in the Fetal Sheep whilst Exposed to Sildenafil Citrate

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Abstract
Introduction: Newborns exposed to sildenafil citrate (SC) in utero have increased rates of persistent pulmonary hypertension. The mechanism behind this has not yet been fully elucidated. We aimed to utilize a combination of clinically relevant MRI techniques to comprehensively characterize the haemodynamics of the fetal sheep whilst under the influence of SC. We hypothesized that these MRI techniques would detect SC-induced increases in pulmonary blood flow and oxygen delivery prior to birth. Methods: At 116–117 days gestational age (term, 150 days), pregnant Merino ewes (\(n=9\)) underwent fetal catheterization surgery. MRI scans were performed during a basal state and then repeated during a constant umbilical vein infusion of SC to measure blood flow and oxygenation within the major vessels of the fetal circulation using phase-contrast-MRI and T\textsubscript{2} oximetry. Results: Right and left ventricular cardiac outputs were not different between states. Pulmonary blood flow increased during the SC state resulting in elevated pulmonary oxygen delivery. Right to left heart shunting through the foramen ovale was reduced without reducing cerebral oxygen delivery. Conclusion: SC induces alterations to pulmonary haemodynamics \textit{in utero}; a characteristic that if maintained may underlie or act as a precursor towards the elevated rates of poor pulmonary outcomes after birth. These MRI techniques are the first to comprehensively characterize sildenafil’s direct impact on the pulmonary vasculature and its indirect detriment to the flow of oxygen-rich blood through the foramen ovale.

Introduction

When the developing fetus fails to reach its genetic growth potential, it is considered growth restricted. This fetal growth restriction (FGR) increases the risk of stillbirth, preterm delivery, longer stays in the neonatal inten-
sive care unit, and perinatal death [1, 2]. Should these growth-restricted fetuses survive the initial hurdles of the perinatal period, they then face the lifelong epigenetically regulated predisposition to the onset of chronic disease [3, 4], which continues to carry a heavy social and economic burden throughout the life course. As such, there is a clinical need for developing both innovative techniques that will allow a higher, more accurate rate of FGR detection/surveillance [5, 6] and intervention strategies that could be applied during gestation to improve fetal growth/development [7–10].

Sildenafil citrate (SC) is a potent vasodilator that regulates vascular tone through the inhibition of phosphodiesterase-5. In recent years, SC has garnered interest in its potential application to the treatment of FGR. This interest was based firstly on the abundance of phosphodiesterase-5 in the placenta that allows SC to enhance nitric oxide-induced vasodilation of the utero-placental vascular beds and secondly on promising results from preclinical and small-scale studies in the human FGR pregnancy [11–13]. From this, the initiation of the multicentre randomized placebo-controlled Sildenafil TheRapy in Dismal prognosis Early-onset fetal growth Restriction (STRIDER) trial ensued. Whilst the UK and Australia/NZ arms showed no observable positive or negative impacts of SC in severe FGR pregnancies [14, 15], the Dutch STRIDER trial was halted at interim analysis due to increased rates of persistent pulmonary hypertension in newborns and elevated, albeit not significant, rates of neonatal death [16, 17]. The mechanism behind these poor outcomes has not yet been fully elucidated; however, the capacity of SC to cross the placenta into the fetal circulation and the persistent vascular alterations in SC-treated neonates has raised concerns that SC may have unintended detrimental consequences on the fetal vasculature instead of an isolated action on the utero-placental circulation [18]. Fully understanding the impact that SC has on the fetal vasculature in utero may provide insight into the poor prognosis of the STRIDER trials and allow for development of benchmarks in future studies investigating alternative FGR interventions.

We have recently utilized preclinical sheep and clinical human studies to validate the use of two advanced MRI techniques to measure blood flow and oxygenation within the fetal circulation [19, 20]. Phase-contrast (PC)-MRI allows for the measurement of blood flow within the fetal circulation and T2 oximetry utilizes the different magnetic properties of oxy- and deoxy-haemoglobin to determine oxygen saturation of the blood. A combination of these techniques allows for a comprehensive characterization of fetal haemodynamics [21, 22].

Herein, we aimed to utilize a combination of the clinically relevant PC-MRI and T2 oximetry to comprehensively characterize the haemodynamics of the normally grown fetal sheep exposed to SC. We hypothesized that these MRI techniques would be able to detect SC-induced increases in pulmonary blood flow (PBF) and oxygen delivery (DO2) prior to birth.

**Materials and Methods**

**Fetal Catheterization Surgery**

At 116–117 days (d) gestational age (GA), Merino ewes (n = 9) underwent aseptic surgery as previously described [23]. Anaesthesia was induced with intravenous diazepam (0.3 mg/kg) and ketamine (5 mg/kg) and then maintained with isoflurane (1.5–2.5%). Vascular catheters were implanted into the maternal jugular vein, fetal femoral vein, umbilical vein, femoral artery, and the amniotic cavity as previously described [23]. The antibiotic and analgesic regimen for both the ewe and her fetus was as previously described and available in the online data supplement (for all online suppl. material, see www.karger.com/doi/10.1159/000526972).

**Experimental Protocol**

Pregnant ewes were anaesthetized as per surgery and underwent MRI scans between 119 and 120 d GA. The fetal femoral artery and amniotic catheters were connected to displacement transducers, a quad-bridge amplifier and a data acquisition unit (PowerLab; ADInstruments, Castle Hill, NSW, Australia) to record fetal blood pressure (corrected for amniotic pressure). All data were sampled at a rate of 1,000 Hz, digitized and recorded using LabChart 7 (ADInstruments).

Imaging was performed on a 3 Tesla clinical MRI system (MAGNETOM Skyra; Siemens Healthineers, Germany). Fetal blood flow measurements and oxygen saturations were determined by PC-MRI and T2 MRI oximetry as previously described [19–21]. Measurements were taken in a basal state and then during an umbilical vein infusion of SC (Sigma-Aldrich, USA). Initial dosing regimens were set to target fetal SC plasma concentrations as previously described [18]. Bolus doses (15–60 µg) and infusion rates (0.0175–0.035 mg/kg/h) of SC were adjusted between animal experiments based on interim plasma SC concentration analysis. MRI acquisition during the SC state began at 15 min into SC infusion.

**Determination of Blood Flow within the Fetal Circulation**

The fetal femoral arterial pressure waveform was used to generate a cardiac trigger for MR imaging, PC imaging was performed to measure blood flow within the fetal circulation with corresponding vessel-appropriate velocity encoding. PC-MRI acquisitions used parameters as per our previously published techniques and available in the online data supplement [20, 24].

**Determination of Oxygen Saturation within the Fetal Circulation**

Vessel T2 oximetry was performed using a T2-prepared pulse sequence with a balanced steady-state free precession acquisition (Myomaps, Siemens) [19]. MRI parameters and analysis details are available in the online data supplement.
Sildenafil Decreases Right to Left Fetal Heart Shunting

**Determination of Oxygen Delivery and Consumption**

Blood flow and T2-derived oxygen saturations were combined to calculate overall fetal oxygen delivery (DO2), fetal oxygen consumption (VO2), cerebral DO2, cerebral VO2, and pulmonary DO2 using equations 1–5 in the online data supplement.

**Blood Sampling and Fetal Blood Gas Measurements**

Fetal arterial blood samples were collected daily to measure the partial pressure of oxygen (PaO2), partial pressure of carbon dioxide (PaCO2), oxygen saturation (SaO2), pH, haemoglobin, haematocrit, base excess, and lactate concentrations, temperature corrected to 39°C for sheep blood with a RAPIDPOINT 500 (Siemens Healthineers, Melbourne, Australia). During the MRI scan, arterial samples for fetal blood gas analysis were taken at the beginning and end of each state and blood samples (2 mL) were collected at 10, 30, 45, and 60 min post-SC administration for subsequent SC plasma concentration analysis.

**Table 1. Fetal blood gases, Hb, and lactate values prior to anaesthesia for MRI and during basal and SC states**

|                     | Pre-anaesthesia (n = 9) | Basal state (n = 9) | SC state (n = 9) | p value |
|---------------------|-------------------------|---------------------|-----------------|---------|
| PaO2 (mm Hg)        | 20.0±1.4a               | 22.2±1.1b           | 20.7±1.6a       | 0.0087  |
| PaCO2 (mm Hg)       | 49.6±3.6a               | 54.2±5.5b           | 56.2±6.3b       | 0.0126  |
| pH                  | 7.386±0.017a            | 7.306±0.032b        | 7.303±0.035b    | <0.0001 |
| SaO2 (%)            | 62.3±4.2a,b             | 64.1±4.5a           | 58.6±7.3b       | 0.0249  |
| Hb (g/L)            | 102.7±7.0               | 99.0±7.5            | 101.4±8.1       | ns      |
| Hct (%)             | 30.2±1.9                | 29.1±2.2            | 29.9±2.5        | ns      |
| Base excess (mmol/L)| 3.5±2.1a                | −0.4±2.3b           | 0.1±1.9b        | <0.0001 |
| Lactate (mmol/L)    | 1.22±0.17a              | 2.14±0.40b          | 2.72±0.51c      | <0.0001 |

Values are mean ± SD. Ewes were anaesthetized and lying on their left side during MRI. Data analysed by repeated-measures one-way ANOVA with Bonferroni’s correction for multiple comparisons. ns, p > 0.05. Superscript alphabetical letters indicate significant differences between timepoints (p < 0.05) such that values with different alphabetical letters are statistically different from each other and values with the same alphabetical letters are not different.

**Fig. 1.** Impact of SC (blue circles, right y-axis) on fetal (n = 9) mean arterial pressure (MAP; pink triangles; left y-axis) and heart rate (HR; green squares; left y-axis) during MRI acquisition of fetal blood flow (PC-MRI) and oxygenation (T2 oximetry) measures. Data analysed by a repeated-measures one-way ANOVA with a Bonferroni correction for multiple comparisons. Data presented as 5-min means ± SD. p ≤ 0.05.
Fig. 2. Effect of SC exposure on umbilical vein blood flow (a), fetal oxygen delivery (DO₂; b), and fetal oxygen consumption (VO₂; c). Data presented as individual data points (n = 9; basal state, unfilled circles; SC state, filled black circles). Data analysed by a paired t test. *Statistically significant difference between basal and SC states. p ≤ 0.05.

Fig. 3. Right ventricular cardiac output (RVCO; a), pulmonary blood flow (PBF; b, e), pulmonary oxygen delivery (c), blood flow through the ductus arteriosus (DA; d) during basal and SC states as well as the relationship between PBF and fetal SC plasma concentrations. SC plasma concentrations obtained from the closest fetal blood sample to the PC-MRI PBF measurement. Data presented as individual data points (n = 9; basal state, unfilled circles; SC state, filled black circles). Data analysed by a paired t test or linear regression analysis. *Statistically significant difference between basal and SC states. p ≤ 0.05.
Determination of SC Fetal Plasma Concentrations
The concentration of SC in fetal plasma was determined using reverse-phase liquid chromatography (see online data supplement).

Post-Mortem
At 123–124 d GA, pregnant ewes were humanely killed with an overdose of sodium pentobarbitone (Virbac, NSW, Australia). The fetus was delivered and weighed.

Statistical Analysis
Blood flow, DO$_2$, and VO$_2$ were analysed by a paired t test (GraphPad Prism version 8, USA). The impact of SC on fetal blood pressure and heart rate (HR) was determined using a repeated-measures one-way ANOVA. Data are presented as mean ± SD and a probability of 5% ($p < 0.05$) was considered significant for all analyses.

Results

Fetal Blood Gas Status
Fetal PaO$_2$ and SaO$_2$ in the SC MRI state were significantly lower (but not outside the normal range of singleton Merino fetuses) compared to the basal MRI state but not the pre-anaesthesia state. pH and base excess were not different between basal and SC MRI states but were both lower than the pre-anaesthesia state (Table 1). There was no difference in Hb and Hct between pre-anaesthesia, basal, and SC states (Table 1). Fetal blood lactate was significantly higher in the basal and SC states than the pre-anaesthesia state but was not different between basal and SC states (Table 1).

Effect of SC on Fetal Blood Pressure and HR
There was no impact of SC exposure on fetal mean arterial pressure or HR (Fig. 1). Fetal SC concentrations were detectable at 10 min after SC administration and throughout the MRI acquisition window (Fig. 1).

Impact of SC on Fetal Oxygen Delivery and Consumption
There was no effect of SC on umbilical vein blood flow (Fig. 2a). Fetal DO$_2$ and VO$_2$ were not different between basal and SC states (Fig. 2b, c).

Effect of SC on PBF and Oxygen Delivery
SC did not impact right ventricular cardiac output (Fig. 3a) but significantly increased PBF (Fig. 3b) and DO$_2$ to the lungs (Fig. 3c). During the SC state, PBF was positively related to the fetal SC plasma concentration such that as fetal SC plasma concentrations increased so did PBF (Fig. 3e). Blood flow through the ductus arteriosus was significantly decreased in the SC state (Fig. 3d).

Effect of SC on Cerebral Blood Flow, Oxygen Delivery, and Consumption
The increased PBF rebalanced how blood flow moved through the heart, manifesting in a decrease in blood flow through the FO (Fig. 4a). However, left ventricular cardiac output (Fig. 4b) and combined carotid artery (Fig. 4c) blood flow were not changed by fetal SC exposure. Fetal cerebral DO$_2$, oxygen extraction fraction (E), and VO$_2$ were not changed by SC exposure (Fig. 4d–f).

Effect of SC on CVO and Blood Flow Distribution
Combined ventricular output (CVO) was not changed by SC (Fig. 5a). Blood flow distribution normalized to CVO was changed such that SC increased PBF with consequent decreases in blood flow distribution through both the ductus arteriosus and FO (Fig. 5b). Visual representations of these blood flow distributions as a percentage of CVO are indicated on Figure 5c and d.

Discussion
Herein we aimed to utilize PC-MRI and T$_2$ oximetry to determine whether fetal exposure to SC would result in changes to PBF and oxygen delivery in utero. We found that a combination of these techniques detected alterations to blood flow and oxygen delivery within the fetal circulation upon a background of this clinically relevant intervention against FGR. SC increased PBF and DO$_2$ without altering the outputs of either the right or left ventricles. Consequently, right to left heart shunting of oxygen-rich blood through the FO was reduced, albeit without detriment to cerebral DO$_2$.

Fetal mean arterial pressure and HR were not different during the basal and SC MRI acquisition windows. Given that SC can transiently alter fetal blood pressure [25], we began MRI acquisitions 15 min after the onset of fetal SC exposure in an attempt to avoid any potential transient haemodynamic instability. Unlike recent findings by De Bie et al. [25], we found no impact of SC on blood pressure, transient or otherwise. It should be noted that fetal SC exposure during the STRIDER trials occurred over a chronic timeframe and thus acute transient alterations to fetal blood pressure or HR at the onset of SC exposure are unlikely to impact longer-term cardiovascular outcomes.

Fetal haemodynamics are sensitive to alterations in fetal oxygenation status [26–28]. Decreased oxygen avail-
ability, as would occur during FGR, leads to a different blood flow distribution pattern than that which occurs when there is an increase in fetal oxygen availability. However, the consequences of each are governed by the fact that during fetal life the LV has two sources of preload: (1) pulmonary venous return and (2) oxygen-rich umbilical vein blood that has been shunted through the FO [29, 30].

In an effort to prioritize oxygen delivery to the brain during fetal hypoxaemia, circulatory changes including decreased PBF and increased dilation of the ductus venosus occur to support an increase in the phenomenon known as “streaming” [31, 32]. Here, oxygen-rich blood returning from the placenta preferentially passes through the ductus venosus, bypassing the liver, on its way towards the right atrium before being shunted through the FO to the left side prior to being pumped towards the brain [33]. In this situation, the proportion of LV preload that is made up of oxygen-rich umbilical vein blood as opposed to deoxygenated blood returning from the lungs increases. In contrast, elevations in fetal oxygenation above in utero physiological levels, such as those that would occur during maternal hyperoxygenation therapy, results in an increase in pulmonary artery dilation with a consequential increase in PBF. As a result, the proportion of LV preload that is made up of deoxygenated pulmonary venous return increases and thus indirectly impairs the ability for maternal hyperoxygenation therapy to have a more significant impact on cerebral DO₂ [34]. Given this oxygen responsiveness of the fetal circulation, the finding in the present study that fetal DO₂ was not different between basal and SC states as well as the lack of SC-induced alterations to fetal HR, blood pressure, carotid or descending aorta blood flow strengthens the case that the

Fig. 4. Effect of fetal SC exposure on blood flow through the foramen ovale (a), left ventricular cardiac output (LVCO; b), combined carotid artery (CCa) blood flow (c), cerebral oxygen delivery (DO₂; d), cerebral oxygen extraction fraction (e), and cerebral oxygen consumption (VO₂; f). Data presented as individual data points (n = 9; basal state, unfilled circles; SC state, filled black circles). Data analysed by a paired t test. *Statistically significant difference between basal and SC states. p ≤ 0.05.
circulatory changes observed were not an indirect effect of SC-induced changes to fetal oxygenation status but rather a direct consequence of SC exposure on the fetal vasculature itself.

SC directly increased both PBF and oxygen delivery. Moreover, PBF was positively correlated with the concentration of SC in the fetal plasma. Concerningly, if sustained the increased PBF and DO\textsubscript{2} may have a negative impact on surfactant protein B expression in the lung [35]. In line with the increased PBF, we also found significantly decreased right to left heart shunting through the FO. This highlights that elevations in PBF due to SC exposure culminates in the creation of a LV preload with less oxygenated blood. However, the altered composition of LV preload was not sufficiently different to significantly decrease cerebral DO\textsubscript{2}.

It should be noted that we performed this study in normally grown normoxaemic fetal sheep. Whilst cerebral DO\textsubscript{2} was not impacted by SC exposure in the present study, the clinical application of SC would be in pregnancies complicated by FGR. The circulatory alterations due to the chronic hypoxaemia associated with

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**Fig. 5.** Effect of SC exposure on combined ventricular output (CVO; a) and blood flow distribution within the fetal circulation (b). Data presented as individual data points (n = 9; basal state, unfilled circles; SC state, filled black circles). c, d Differences in blood flow distribution, as a percentage of CVO, due to SC exposure are indicated by blue/red circles on diagrammatic representations of the fetal circulation. Data analysed by a paired t test. *Statistically significant difference between basal and SC states. p ≤ 0.05.
FGR causes the FGR fetus to be more reliant on the preferential streaming of well-oxygenated blood through the DV to the right side of the heart and then right to left heart shunting through the FO to maintain cerebral DO₂. If the present study were performed in a pregnant sheep model of FGR, it is likely that SC may have directly interfered with the FGR fetus’ endogenous attempt at maintaining cerebral DO₂. Whilst the SC-induced blood flow pattern is similar to that observed when the fetus is hyperoxygenated, unlike the fetal hyperoxygenation scenario the reduction in right to left heart shunting is not offset by the elevated oxygen saturation of the blood. This may suggest that FGR fetuses exposed to SC may be impacted to a greater extent than their normally grown counterparts, particularly if maternal SC treatment does not substantially increase utero-placental perfusion [36]. This hypothesis corresponds with previous work where sheep-equivalent STRIDER-like dosing of SC in a sheep model of FGR found SC exacerbated the growth restriction and directly acted on the fetal vasculature to impair the growth-restricted fetuses normal ability to redistribute blood flow away from the periphery [37]. Certainly, an MRI assessment of blood flow and DO₂ within the circulation of the growth-restricted fetus in response to SC will yield more clinically informative results.

In this study, we utilized a well-established chronically instrumented sheep model of human pregnancy in combination with clinically translatable MRI techniques. As such, anaesthesia of the ewe was a necessary requirement during the MRI sessions. Whilst anaesthetic agents can impact the cardiovascular system, we have previously performed a comparative study that identified isoflurane as the anaesthetic agent that would have the least impact on the fetal cardiovascular system [38] and subsequently utilized this protocol to assess the impact that vasoactive molecules have on the fetal haemodynamics [21]. Moreover, the depth of anaesthesia remained stable across the experimental timeframe and thus the impact of anaesthesia on the fetal circulation is likely to be similar in the basal and SC state.

The SC dosing regime in the present study targeted fetal sheep plasma SC concentrations after SC doses equivalent to those administered in the STRIDER trial were given to pregnant ewes [18,37]. However, the capacity for SC to cross the sheep placenta appears to be limited [25] with an ex vivo dually perfused human cotyledon model suggesting that the transfer capacity of SC across the human placenta may be more efficient [39]. This would result in the human fetus being exposed to a higher concentration of SC during the STRIDER trials than the fetal sheep in this study were exposed to during the MRI acquisitions. Although this limits our ability to directly align the results herein with the in utero cardiovascular alterations that may have occurred in the circulation of fetuses enrolled in the STRIDER trials, we suggest that this may in fact be a strength of the present study and evidence for further cause for concern in the use of SC to treat pregnancy complications antenatally. It is possible that the magnitude of change in right to left heart shunting of human fetuses could be higher than in the fetal sheep due to their potentially higher exposure to SC. This is particularly concerning given that we have previously compared the circulations of the human fetus to the fetal sheep and found that the human fetus has a higher oxygen gradient between blood in the AAo and the MPA [19]. This indicates that the human fetus may already have a higher reliance on “streaming” to maintain cerebral DO₂ and it is therefore possible that a lower exposure of SC may have a detrimental impact.

We have utilized a combination of PC-MRI and T₂ oximetry to comprehensively characterize the impact that SC exposure has on the fetal circulation. SC indirectly reduced right to left heart shunting of oxygen-rich blood by increasing PBF. This alteration to the composition of LV preload blood did not cause a significant decrease in cerebral DO₂ of the fetal sheep. However, it is possible that a combination of being exposed to higher SC concentrations during the STRIDER trials and a species/FGR-induced greater reliance on “streaming” to supply the brain with oxygen would cause the human fetus to be at a greater risk. That being said, there is recent evidence from fetal sheep supported ex utero on an EXTrauterine Environment for Neonatal Development (EXTEND) device showing that SC-induced alterations to fetal haemodynamics are self-limiting with a return to baseline after 8 h post-SC exposure [40]. Therefore, future serial MRI studies assessing fetal haemodynamics over a more chronic timeframe are warranted.

To the best of our knowledge, this is the first study to utilize PC-MRI and T₂ oximetry to assess the impact that a clinically relevant intervention against FGR has on the fetal circulation. However, the findings herein add further evidence for the caution of SC as an intervention against FGR and that the SC-induced increases in PBF may be predictive of a “rebound” pulmonary vasoconstriction and PPH afterbirth when SC exposure is removed. Future studies assessing the relationships between fetal PBF, molecular alterations in the pulmonary vasculature, and neonatal pulmonary outcomes after a
more a more chronic duration of SC exposure than the present study would further elucidate the mechanisms by which SC influences pulmonary outcomes.

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Statement of Ethics

All experimental protocols were reviewed and approved by the Animal Ethics Committee of the South Australian Health and Medical Research Institute (SAHMRI; reference SAM389.19) and abide by the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes developed by the National Health and Medical Research Council. All investigators understood the ethical principles outlined in Grundy et al. [41] and the principles of the 3Rs, specifically the reduction of the use of animals in research.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

Conception or design of the work: Jack R.T. Darby, Christopher K. Macgowan, Mike Seed, and Janna L. Morrison. Acquisition or analysis or interpretation of data for the work: Jack R.T. Darby, Georgia K. Williams, Steven K.S. Cho, Brahmdeep S. Saini, Ashley S. Meakin, Stacey L. Holman, Megan Quinn, Michael D. Wiese, Christopher K. Macgowan, Mike Seed, and Janna L. Morrison. Drafting the work or revising it critically for important intellectual content: Jack R.T. Darby, Brahmdeep S. Saini, Stacey L. Holman, Michael D. Wiese, Christopher K. Macgowan, Mike Seed, and Janna L. Morrison. Final approval of the version to be published and agreement to be accountable for all aspects of the work: all.

Data Availability Statement

All data generated or analysed during this study are included in this article and its online supplementary material. Further enquiries can be directed to the corresponding author on reasonable request.
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