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Antenatal sildenafil treatment improves neonatal pulmonary hemodynamics and gas exchange in lambs with diaphragmatic hernia

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Short title: Antenatal sildenafil for diaphragmatic hernia

Key Words

Congenital abnormalities, fetal therapy, neonatal transition, pulmonary vascular development, pulmonary hypertension

Contribution

What does this work add to what is already known?

Antenatal sildenafil treatment improves neonatal pulmonary haemodynamics and gas exchange in a lamb model of congenital diaphragmatic hernia (CDH), which correlates with previous rodent work demonstrating that antenatal sildenafil treatment improved pulmonary vascular structure.

What are the clinical implications of this work?

Antenatal sildenafil treatment should be investigated in a clinical trial for fetuses with CDH, in order to reduce the incidence and/or severity of persistent pulmonary hypertension of the newborn.

Abstract

Objectives

Infants with congenital diaphragmatic hernia (CDH) are predisposed to pulmonary hypertension after birth, due to lung hypoplasia that impairs fetal pulmonary vascular development. Antenatal sildenafil treatment attenuates abnormal pulmonary vascular and alveolar development in rabbit and rodent CDH models, but whether this translates to functional improvements after birth remains unknown. We aimed to evaluate the effect of antenatal sildenafil on neonatal pulmonary haemodynamics and lung function in lambs with a diaphragmatic hernia (DH).

Methods

A DH was surgically created at ≈80 days gestational age (GA; term≈147d) in 16 fetal lambs. From 105d GA, ewes received either sildenafil (0.21 mg/kg/hr intravenously) or saline infusion. At ≈138d GA, all lambs were instrumented and then delivered via caesarean section. Lambs were ventilated for 120 min with continuous recording of physiological (pulmonary and carotid artery blood flows and pressures; cerebral oxygenation) and ventilatory parameters, and regular assessment of arterial blood gas tensions.

Results

Lung-to-body-weight ratio (0.016 ± 0.001 vs. 0.013 ± 0.001, p=0.06) and dynamic lung compliance (0.8 ± 0.2 vs. 0.7 ± 0.2 mL/cmH2O, p=0.72) were similar in DH-sildenafil lambs (n=6) and DH-saline controls (n=6). Pulmonary vascular resistance decreased following lung aeration to a greater degree in DH-sildenafil lambs and

was 4-fold lower by 120 min after cord clamping (0.6 ± 0.1 vs. 2.2 ± 0.6 mmHg/(L/min); *p*=0.002). Pulmonary arterial pressure was also lower (46 ± 2 vs. 59 ± 2 mmHg; *p*=0.048) and pulmonary blood flow higher (25 ± 3 vs. 8 ± 2 mL/min/kg; *p*=0.02) in DH-sildenafil compared to DH-saline lambs. Throughout the 120 min ventilation period, $PaCO_2$ tended to be lower in DH-sildenafil lambs (63 ± 8 vs. 87 ± 8 mmHg, p=0.057), and there was no significant difference in PaO_2.

Conclusions

Sustained maternal antenatal sildenafil infusion reduced pulmonary arterial pressure and increased pulmonary blood flow in DH lambs for the first 120 min after birth. These findings of improved pulmonary vascular function are consistent with improved pulmonary vascular structure seen in two previous animal models. The data support the rationale for a clinical trial with antenatal sildenafil to reduce the risk of neonatal pulmonary hypertension in infants with CDH.

Introduction

In congenital diaphragmatic hernia (CDH), abdominal organs herniate into the thorax during embryogenesis, interfering with fetal lung development and resulting in severe pulmonary hypoplasia. While CDH is considered a rare disease, it continues to contribute more than 1% of the infant mortality rate in the United States.[1, 2] This is despite more recent advances in antenatal diagnosis and neonatal care at birth and primarily relates to the degree of lung hypoplasia that affects both airway and vascular development.[3] CDH infants are born with small stiff lungs with a reduced gas-exchange surface area and fewer pulmonary vessels with altered vasoreactivity.[4, 5] These changes then predispose infants to respiratory insufficiency and pulmonary hypertension immediately after birth.

Early attempts to surgically repair the diaphragmatic defect *in utero* were unsuccessful, so subsequent fetal interventions have focused instead on enhancing prenatal lung growth.[6, 7] This approach is feasible due to improved antenatal detection during obstetric morphology ultrasound examinations, during which the severity of abnormal lung development can be quantified.[8] One potential strategy to improve lung growth is by increasing the volume of lung liquid retained within the future airways by occluding the fetal trachea.[9-11] The clinical evolution of this technique, fetoscopic endoluminal tracheal occlusion (FETO[12]), has shown some promising preliminary outcomes, with two randomised clinical trials ongoing in CDH fetuses at risk for severe and moderate lung hypoplasia (Tracheal Occlusion to Accelerate Lung growth, TOTAL).[13-16] Nevertheless, despite an apparent increase in

survival, approximately half of infants with severe lung hypoplasia do not survive and there is limited benefit regarding the incidence of persistent pulmonary hypertension of the newborn (PPHN).[17-19] Hence, antenatal therapies that specifically address the pulmonary vascular abnormalities associated with CDH appear necessary.[20-22]

PPHN in CDH infants is often refractory to treatment with inhaled nitric oxide (NO), suggesting abnormalities in endothelium dependent vasodilatory pathways.[23] This is supported by the finding that phosphodiesterase-5 (PDE5) is upregulated in neonatal CDH lungs; PDE5 suppresses NO-mediated vasodilation by rapidly degrading cyclic guanosine monophosphate (cGMP).[24] Furthermore, antenatal treatment with the PDE5 inhibitor sildenafil attenuates pulmonary vascular abnormalities in both a toxic rodent CDH model and a surgical rabbit CDH model, which is more clinically relevant in terms of lung development.[25-33] In these animal studies, antenatal sildenafil treatment increases cGMP and vascular endothelial growth factor (VEGF) expression in lung tissue, increases distal pulmonary vessel density, decreases pulmonary vascular muscularisation, reduces right ventricular wall thickness, and increases distal airway complexity.[25-33] However, it is unclear if these improvements in pulmonary vascular structure and biochemistry translate to improved pulmonary haemodynamics after birth. Our aim was to investigate the physiological effects of antenatal sildenafil on pulmonary haemodynamics and lung function during the neonatal transition in lambs with diaphragmatic hernia (DH).

Methods

Animal model

General surgical methods

This experiment was performed in accordance with guidelines established by the National Health and Medical Research Council of Australia and was approved by the Monash University animal ethics committee. For all surgical procedures, eight Merino X Border-Leicester ewes carrying twin pregnancies were anaesthetised with an intravenous bolus of sodium thiopentone (1 g in 20 mL; Pentothal, Jurox, New Zealand), intubated with an 8 mm endotracheal tube (Portex Ltd., Kent, England), and maintained with inhaled isoflurane (≈2% in room air/oxygen; Isoflow, Abbot) administered via a positive pressure ventilator (EV500 Anaesthesia Ventilator, ULCO Medical Engineering, NSW, Australia). End-tidal CO2, tidal volume, heart rate and oxygen saturation were continuously monitored (SurgiVet Vital Signs Monitor, Smiths Medial, USA), along with the absence of a corneal reflex to ensure adequate analgesia and maternal wellbeing.

Diaphragmatic hernia creation

At ≈80 days gestational age (dGA; term≈147 dGA), a diaphragmatic hernia (DH) was surgically created in 16 fetal lambs, as previously described.[34] Briefly, maternal midline laparotomy and hysterotomy allowed the fetal chest to be exposed and incised through the ninth intercostal space. A small segment of the fetal diaphragm was excised and the stomach and bowels were pulled into the thoracic cavity. The

amniotic fluid content was restored and 1000 mg Cefazolin was administered directly into the amniotic sac as antibiotic prophylaxis (Cefazolin-AFT 1000 mg in 5 mL sterile water, AFT Pharmaceuticals Pty. Ltd., Australia). Ewes received three days of post-operative analgesia (transdermal fentanyl patch, 75 µg/hr; Janssen Cilag) and were monitored daily until delivery at 138d GA.

Sildenafil infusion

At 103d GA, catheters were placed in the maternal right jugular vein and carotid artery under general anaesthesia. The jugular venous catheter was connected to an infusion pump (CADD-Legacy 1 Ambulatory Infusion Pump Model 6400; Smiths Medical Australasia Pty. Ltd., Australia), which was secured to the ewe's back using netting (Tubular-Net, Sutherland Medical Pty. Ltd., Australia).

At 105d GA (equivalent to human fetal lung development at 22 weeks of gestation), ewes carrying twins were allocated to either DH-sildenafil or DH-saline. In four ewes (n=8 fetuses; DH-sildenafil), the infusion pump administered sildenafil via continuous intravenous infusion at a dose of 0.21 mg/kg/hr until delivery (2.8 mg/mL sildenafil citrate in 0.9% sodium chloride, Fagron, Belgium). In four ewes (n=8 fetuses; DH-saline), the infusion pump administered an equivalent volume of 0.9% sodium chloride.

Delivery and ventilation

At ≈138 dGA (near-term), fetal lambs were instrumented prior to caesarean delivery as previously described.[34] Briefly, fetal lambs were exteriorised and intubated with a clamped endotracheal tube (size 4.0). Polyvinyl catheters were placed in the left carotid artery and main pulmonary artery to allow continuous arterial pressure monitoring, and in the left jugular vein to allow neonatal drug administration if required. Ultrasonic flow probes (Transonic Systems, Ithaca, NY, USA) were placed around the right carotid and left pulmonary artery, to continuously record blood flow. A pulse-oximeter (Masimo, Radical 7, CA, USA) was placed on the right forelimb, near-infrared spectroscopy probe (NIRS; Casmed Foresight, CAS Medical Systems Inc., Branford, CT, USA) on the forehead, and temperature probe in the rectum. The stomach was drained via an orogastric tube. After instrumentation, lambs were delivered from the uterus and the endotracheal tube was unclamped.

The umbilical cord was immediately clamped and ventilation commenced with a 30 sec sustained inflation (35 cmH_2O, 21 % O_2) followed by intermittent positive pressure ventilation (iPPV) in volume guarantee mode using a target tidal volume of 4 mL/kg (Babylog 8000, Dräger, Lübeck, Germany), peak inspiratory pressure (PIP) limit of 35 cmH_2O, and positive end-expiratory pressure (PEEP) of 5 cmH_2O. If the target tidal volume was not reached after 30 sec, a second sustained inflation was performed followed by iPPV for a total of 120 min.

Lambs were moved to a warming bed (CosyCot, Fisher and Paykel, Auckland, New Zealand) and sedated with alfaxalone (10 mg/kg/hr; Alfaxan, Jurox). Respiratory support was titrated to achieve $PaCO_2$ 60-80 mmHg, PaO_2 >40 mmHg and oxygen

saturation (SaO_2) 85-88 %, as previously described.[34] Lambs were euthanised (sodium pentobarbitone i.v. 100 mg/kg) after completing the 120 min ventilation protocol or earlier due to ethical endpoints (i.e. severe acidosis or pneumothorax).

Outcome measures

Plasma sildenafil concentration

Maternal arterial blood samples were obtained at 1, 7 and 21 days after the antenatal infusion commenced. At delivery, paired maternal and fetal arterial blood samples were obtained. Plasma from these samples was isolated via centrifugation (3000 revolutions per minute for 10 min). Total sildenafil concentration (calculated as sildenafil concentration + 50% of its active metabolite *N*-desmethyl-sildenafil[35]) was analysed using ultra high performance liquid chromatography (Vanquish Flex Quaternary, ThermoFisher Scientific, Australia) in tandem with mass spectrometry (TSQ Quantiva, ThermoFisher Scientific, Australia).

Neonatal cardiopulmonary haemodynamics and lung function

Pulmonary and carotid arterial blood flows and pressures, cerebral tissue oxygen saturation ($SctO_2$), tidal volume and airway pressures were continuously recorded using LabChart (ADInstruments, NSW, Australia) and analysed offline in 20 sec epochs. Arterial blood gas tensions were assessed every 5 min during the first 30 min of ventilation and every 10 min thereafter.

Pulmonary vascular resistance (PVR), dynamic lung compliance, alveolar-arterial difference in oxygen tension ($AaDO_2$) and cerebral oxygen delivery (DO_2) were calculated using equations listed in Table 1.

Post-mortem examination

Presence of a diaphragmatic defect and herniation of visceral organs was confirmed during post-mortem examination. Both lungs were weighed and expressed as a ratio to the body weight (fresh lung-to-body weight ratio; LBWR).

Statistical analysis

We have previously shown that pulmonary blood flow (PBF) in sham-operated controls is 2.4-fold greater than in DH lambs.[34] Based on the variability in that study, we determined that at least six successful animals per group would provide power of ≥ 80% with a two-sided type I error of 5% to detect a 1.5-fold increase in PBF in DH-sildenafil lambs compared to DH-saline lambs. The Shapiro-Wilk test was used to assess frequency distributions. Normally distributed data are expressed as means ± standard error of the mean, and data that is not normally distributed is presented as medians (interquartile range). For physiological data, differences between DH-sildenafil and DH-saline lambs were analysed over time and between groups using two-way repeated measures ANOVA with post-hoc analysis (Holm-Sidak) determining the time that differences were evident (SigmaPlot v13.0, Systat Software Inc.). Statistical significance was accepted when $p<0.05$.

Data from historical controls (no DH, ventilated using the same protocol) are displayed in figures to provide physiological context for the ovine model, however no statistical comparisons with these historical controls are made.[36]

Results

Plasma sildenafil concentrations

Maternal plasma sildenafil concentration was 68 ± 20 ng/mL after 24 hrs of continuous intravenous infusion, 69 ± 17 ng/mL after 7 days and 79 ± 9 ng/mL after 21 days. Immediately prior to delivery (≈33 days after the infusion began), maternal plasma sildenafil concentration was 118 ± 50 ng/mL and fetal plasma sildenafil concentration was 3 ± 0.5 ng/mL. After 120 min of ventilation, neonatal plasma sildenafil concentration remained at 3 ± 0.5 ng/mL. Sildenafil was not detected in maternal or fetal plasma samples from DH-saline animals.

Animal groups and gross morphology

14 (of 16) fetuses survived to delivery; 7 DH-sildenafil lambs (88%) and 7 DH-saline (88%). All DH-sildenafil lambs survived the 120 min neonatal ventilation, however it was necessary to humanely euthanise one DH-saline lamb at 90 min after birth due to developing treatment-resistant pneumothorax. Only lambs with a confirmed diaphragmatic defect at post-mortem examination were included in the analysis (6 DH-sildenafil lambs and 6 DH-saline lambs).

Body weight was not significantly different between DH-sildenafil and DH-saline lambs (4.14 ± 0.25 vs. 4.49 ± 0.34 kg, p=0.43). LBWR was not significantly different between DH-sildenafil and DH-saline lambs (0.016 ± 0.001 vs. 0.013 ± 0.001, p=0.06).

Ventilation parameters and arterial blood gas tensions

Peak inspiratory pressures and positive end-expiratory pressures were not significantly different between groups throughout the 120 min ventilation period (Figure 1A). However, tidal volumes (V_T) were significantly greater in DH-sildenafil lambs compared to DH-saline lambs at 15 min after cord clamping (4.1 ± 0.5 vs. 2.5 ± 0.5 mL/kg, *p*=0.03; Figure 1B). At the end of the 120 min ventilation period, there was no significant difference in V_T (4.1 ± 0.4 vs. 3.7 ± 0.5 mL/kg, *p*=0.45).

PaO_2 and SaO_2 increased more rapidly after cord clamping in DH-sildenafil lambs. At 10 min after cord clamping both PaO_2 (57 ± 18 vs. 24 ± 5 mmHg, *p*=0.02) and SaO_2 (80 ± 8 vs. 44 ± 12 %, *p*=0.009) were greater in DH-sildenafil than DH-saline lambs, despite similar FiO_2 (62 ± 14 vs. 76 ± 11 %, *p*=0.48). However, after 15 mins the PaO_2 and SaO_2 were no longer significantly different (Figure 2A and 2B). $AaDO_2$ was not significantly different between DH-sildenafil and DH-saline lambs throughout the 120 min ventilation period (288 ± 84 vs. 308 ± 87 mmHg, *p*=0.88), nor was the oxygenation index (23 ± 10 vs. 33 ± 10, *p*=0.46).

Mean $PaCO_2$ tended to be lower in DH-sildenafil lambs compared to DH-saline lambs throughout the 120 min ventilation period (63 ± 8 vs. 87 ± 8 mmHg, *p*=0.057; Figure 2C). As minute volume and $PaCO_2$ are inversely related, we calculated the product of the two (MV x $PaCO_2$) to help explain any differences in $PaCO_2$ levels. This product was initially not different between the groups, which demonstrates that lower $PaCO_2$ in DH-sildenafil lambs was associated with better ventilation. However, by 60 min after cord clamping MV x $PaCO_2$ was significantly lower in DH-sildenafil

lambs compared to DH-saline lambs (49 ± 8 vs. 96 ± 24 mL·bpm·mmHg, p=0.045), which demonstrates that $PaCO_2$ was lower in DH-sildenafil lambs despite similar ventilation. pH was not significantly different between groups throughout the 120 min ventilation period (7.22 ± 0.05 vs. 7.11 ± 0.06, p=0.18, Figure 2D).

Pulmonary perfusion

Pulmonary blood flow (PBF) increased to a greater degree after cord clamping in DH-sildenafil compared to DH-saline lambs (Figure 3A), and at 20 min after cord clamping PBF was 2-fold greater (42 ± 4 vs. 22 ± 3 mL/min/kg, p=0.006). By the end of the 120 min ventilation period, PBF was 3-fold greater in DH-sildenafil compared to DH-saline lambs (25 ± 3 vs. 8 ± 2 mL/min/kg, p=0.02). Pulmonary arterial pressure increased rapidly after cord clamping in both groups, however to a lesser degree in DH-sildenafil lambs (Figure 3B). By the end of the 120 min ventilation period, pulmonary arterial pressure was significantly lower in DH-sildenafil compared to DH-saline lambs (46 ± 2 vs. 59 ± 2, p=0.048).

Pulmonary vascular resistance (PVR) was lower in DH-sildenafil lambs before cord clamping (3.2 ± 0.7 vs. 6.5 ± 0.9 mmHg/(mL/min), p<0.001) and throughout the 120 min neonatal ventilation (Figure 3C). The magnitude of the difference in PVR between DH-sildenafil and DH-saline lambs increased between 20 min (0.33 ± 0.08 vs. 0.71 ± 0.08 mmHg/(mL/min), p=0.007), 60 min (0.45 ± 0.08 vs. 1.86 ± 0.54

mmHg/(mL/min), *p*=0.008) and 120 min (0.60 ± 0.13 vs. 2.17 ± 0.58 mmHg/(mL/min), *p*=0.002) after cord clamping. PBF at the end of diastole (EDF) was negative in both DH-sildenafil and DH-saline lambs *before* cord clamping (-14.2 ± 4.2 vs. -8.7 ± 1.3, *p*=0.46), and became positive within 10 min after cord clamping (14.4 ± 6.1 vs. 7.7 ± 3.2, *p*=0.179). In DH-saline lambs, EDF subsequently decreased during the neonatal ventilation and ultimately returned to ~0 mL/min/kg by 120 min (Figure 3D). In contrast, EDF remained positive in DH-sildenafil lambs and at the end of the 120 min neonatal ventilation was significantly greater than in DH-saline lambs (10.2 ± 1.6 vs. 0.6 ± 1.3 mL/min/kg, *p*=0.036).

Cerebral perfusion and oxygenation

Carotid arterial blood pressure (CAP) and blood flow (CBF) rapidly increased with 5 min after cord clamping in both groups of lambs, although the increase was smaller in DH-sildenafil lambs (Figure 4A and 4B). At 10 min after cord clamping, DH-sildenafil lambs had a ~20% lower CAP (61 ± 6 vs. 78 ± 4 mmHg, *p*=0.046) and ~33% lower CBF (20.4 ± 4.5 vs. 32.9 ± 4.9 mL/min/kg, *p*=0.042) compared to DH-saline lambs. From 10 to 120 min after cord clamping, both CAP and CBF gradually decreased in both groups. At the end of the experimental period, both CAP (49 ± 6 vs. 56 ± 6 mmHg, *p*=0.42) and CBF (12.4 ± 2.0 vs. 14.3 ± 2.8 mL/min/kg, *p*=0.78) were similar in DH-sildenafil and DH-saline lambs.

Heart rate was lower in DH-sildenafil lambs compared to DH-saline lambs between 5 min (141 ± 10 vs. 181 ± 11, *p*=0.02) and 10 min after cord clamping (153 ± 15 vs.

202 ± 16 bpm, *p*=0.005), but the heart rate was not significantly different at 20 min after cord clamping (152 ± 9 vs. 169 ± 12 bpm, *p*=0.34) or thereafter (Figure 4C).

Cerebral oxygen delivery (DO_2) was greater in DH-sildenafil lambs than in DH-saline lambs at 5 min after cord clamping (1.7 ± 0.4 vs. 0.6 ± 0.2 mL/min/kg, *p*=0.04), but was not significantly different at 10 min after cord clamping (2.6 ± 0.6 vs. 2.5 ± 0.8 mL/min/kg, *p*=0.89) or thereafter.

Cerebral tissue oxygen saturation ($SctO_2$) decreased rapidly in both groups after cord clamping, but recovered more rapidly in DH-sildenafil lambs (Figure 4D). $SctO_2$ was significantly greater in DH-sildenafil lambs than in DH-saline lambs between 7 and 14 min after cord clamping (*at 10 min* 65 ± 5 vs. 41 ± 7 %, *p*=0.007). At the end of the experimental period, $SctO_2$ was not significantly different between groups (65 ± 4 vs. 55 ± 10 %, *p*=0.18).

Discussion

We have demonstrated that antenatal sildenafil treatment causes a greater reduction in PVR after birth in DH lambs, resulting in higher PBF with lower pulmonary arterial pressures.

As we have previously shown, DH lambs experience severe hypoxia at birth if the umbilical cord is immediately clamped.[37] This is because the hypoplastic lungs of DH lambs are slow to aerate and perfuse at birth, so cannot take over the role of gas exchange from the placenta as rapidly as age-matched control lambs. Our sildenafil treated DH lambs also experienced a hypoxic period immediately after birth, however, as tidal volumes and PBF increased more rapidly, cerebral oxygenation recovered considerably faster.

Antenatal sildenafil treated DH lambs maintained higher PBF throughout the 120 min ventilation period despite reduced pulmonary arterial pressures, which reflects a considerably lower PVR. PVR was lower in DH-sildenafil lambs during the fetal instrumentation period immediately before birth. This is consistent with previous studies in rabbits[33], which was attributed to an improvement in fixed, structural factors such as the density of distal pulmonary blood vessels.[25, 29, 31, 33] However, the magnitude of the difference in PVR between DH-sildenafil and DH-saline lambs increased after birth, which may reflect a greater vasodilatory response to lung aeration and on-going ventilation. The mechanisms involved are unknown, but may include decreased muscularisation of pulmonary arteries and increased expression of vasodilatory mediators, as shown in rodent DH models.[25, 26, 28, 29, 33] Alternatively,

antenatal sildenafil may enhance the vasodilatory response to birth-related stimuli such as increased oxygenation.[38]

Despite higher PBF, O_2 gas exchange was not different between the two DH groups during the 120 min neonatal ventilation period. However, $PaCO_2$ could more easily be maintained within or below the permissive hypercapnia range (60 – 80 mmHg), reflected by a significantly lower MV x $PaCO_2$ product and indicating improved CO_2 gas exchange in antenatal sildenafil treated DH lambs. This inconsistency in improvements between O_2 and CO_2 exchange likely reflects the higher solubility and hence greater diffusion capacity of CO_2 across the alveolar-capillary membrane.[39] Antenatal sildenafil therapy increases distal airway complexity and gas exchange surface area in rodent DH models, which combined with higher PBF, may have improved CO_2 exchange without being sufficient to significantly improve O_2 exchange.[26, 29, 30, 33]

Reverse (away from lungs) flow in the pulmonary arteries during diastole is a common feature of PBF before birth, resulting in continuous right-to-left shunting via the ductus arteriosus throughout the cardiac cycle. After lung aeration, the large decrease in PVR rapidly changes the PBF waveform. Reverse PBF is abolished and forward flow into the lung becomes continuous, resulting in positive end-diastolic PBFs (EDF). In untreated DH lambs, EDF was lower and returned to near 0 towards the end of the ventilation period (Figure 3D), suggesting that right-to-left shunting, a common feature of PPHN, may have soon re-emerged. In contrast, in DH lambs

treated with sildenafil, higher EDF and a lower PVR make it less likely that right-to-left shunting would re-emerge.

In rodent models, the effect of antenatal sildenafil on gross lung size is unclear. Most have found no difference[25, 29, 33, 40], one reported a decrease[27] whereas two studies have reported an increase in lung weight.[26, 30] While there was no significant difference, we found that DH lambs treated with sildenafil tended (p=0.06) to have greater lung-to-body weight ratios than untreated lambs, but the lungs were still markedly hypoplastic in both treated (48% of control) and untreated (39%) DH lambs.[34] Indeed, while dynamic lung compliance was initially (during the first 20 min) better, by the end of the 120 min ventilation period it was no longer different. These findings suggest that antenatal sildenafil treatment does not improve lung parenchymal growth to the same degree as it improves pulmonary vascular development and function. Hence, combining antenatal sildenafil therapy with FETO, which significantly improves lung growth but has limited effect on the pulmonary vasculature, could provide synergistic benefit for fetuses with severe CDH, as demonstrated in the rabbit CDH model.[41]

We focused primarily on the physiological effects of antenatal sildenafil treatment, so our study is limited by the absence of histological evidence to confirm the morphological and biochemical features underlying the observed physiology. However, the effects of sildenafil on pulmonary vascular development have been well described in other animal models of CDH and correlate well with our current findings.[25-33] Interestingly, fetal plasma sildenafil concentrations in our lambs

immediately prior to delivery (2 – 5 ng/mL), were similar to those obtained in rats by Mous *et al.*[26], but lower than those obtained by Luong *et al*.[25] and in rabbits by Russo *et al*.[33] However, as plasma concentrations of sildenafil persisted in the same range until the end of the ventilation experiment, residual effects of sildenafil on the pulmonary arterial pressure and PVR may have confounded our results.[42] However, the magnitude of the reduction in PVR seen in our study (360%) is significantly greater than when sildenafil is only administered after birth to the neonate (18%).[42] Thus, in future studies it will be important to clarify whether the observed benefits of sildenafil persist once it has been cleared from the circulation.

Another limitation of our study is that we did not investigate the effect of antenatal sildenafil treatment on lambs without a DH. When sildenafil was given to rodents and rabbits without a DH, it decreased the number of distal vessels[25, 33] and decreased total vascular volume.[26] Given the recent safety concerns regarding the use of antenatal sildenafil to treat intrauterine growth restriction (STRIDER[43, 44]), these concerning findings in rodents and rabbits may require further investigation in the lamb DH model before antenatal sildenafil treatment is considered for mild and moderate CDH cases that may not have significantly abnormal pulmonary vascular development.

In conclusion, we have shown that antenatal sildenafil treatment improves neonatal pulmonary haemodynamics in the lambs with DH. These promising findings correlate with previous work demonstrating that sildenafil attenuates abnormal pulmonary vascular development in DH, and provide evidence for investigating sildenafil in a

clinical setting for fetuses with severe CDH, as is currently underway in Belgium and France.[45]

Acknowledgements

We would also like to acknowledge the technical assistance of Dr Ilias Nitsos, Valerie Zahra, and Dalibor Stanojkovic.

Funding Sources

This research project was funded by grants from the Royal Australian and New Zealand College of Obstetricians and Gynaecologists (RANZCOG) Foundation, Cabrini Foundation, CDH Australia, and the Victorian Government's Operational Infrastructure Support Program. These funders were not involved in the study design; in the collection, analysis and interpretation of the data; in the writing of the report; or in the decision to submit the paper for publication.

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Figure Legends

Figure 1: (A) Peak inspiratory pressure (PIP; cmH_2O) and (B) tidal volume (V_T; mL/kg) during the 120 min ventilation after cord clamping in lambs with a diaphragmatic hernia (DH) that received sildenafil (DH-sildenafil; white squares, n=6) and saline (DH-saline; black squares, n=6). Two-way repeated measures ANOVA (group, time) with Holm-Sidak's multiple comparisons test. * $p<0.05$ for effect of treatment at each timepoint below line. Data from historical controls (no DH; grey circles, n=6) displayed to provide physiological context for the ovine model, however no statistical comparisons with these historical controls were made.

Figure 2: (A) Partial pressure of arterial oxygen (PaO_2; mm Hg), (B) arterial oxygen saturation (SaO_2; %), (C) partial pressure of arterial carbon dioxide ($PaCO_2$; mm Hg) and (D) arterial pH during the 120 min ventilation after cord clamping in lambs with a diaphragmatic hernia (DH) that received sildenafil (DH-sildenafil; white squares, n=6) and saline (DH-saline; black squares, n=6). Two-way repeated measures ANOVA (group, time) with Holm-Sidak's multiple comparisons test. * $p<0.05$ for effect of treatment at each timepoint below line. Data from historical controls (no DH; grey circles, n=6) displayed to provide physiological context for the ovine model, however no statistical comparisons with these historical controls were made.

Figure 3: (A) Pulmonary arterial blood flow corrected for body weight (PBF; mL/min/kg), (B) pulmonary arterial blood pressure (PAP; mm Hg), (C) pulmonary

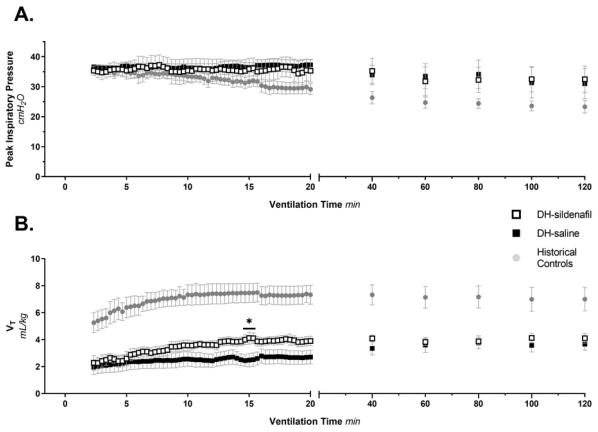
vascular resistance (PVR; mm Hg/(mL/min)) presented on a logarithmic (base 2) scale, and (D) end-diastolic pulmonary blood flow (EDF; mL/min/kg) during the 120 min ventilation after cord clamping in lambs with a diaphragmatic hernia (DH) that received sildenafil (DH-sildenafil; white squares, n=6) and saline (DH-saline; black squares, n=6). Two-way repeated measures ANOVA (group, time) with Holm-Sidak's multiple comparisons test. * $p<0.05$ for effect of treatment at each timepoint below line. Data from historical controls (no DH; grey circles, n=6) displayed to provide physiological context for the ovine model, however no statistical comparisons with these historical controls were made.

Figure 4: (A) Carotid arterial blood pressure (mm Hg), (B) carotid arterial blood flow corrected for body weight (mL/min/kg) (C) heart rate (bpm), and (D) cerebral tissue oxygen saturation ($SctO_2$) during the 120 min ventilation after cord clamping in lambs with a diaphragmatic hernia (DH) that received sildenafil (DH-sildenafil; white squares, n=6) and saline (DH-saline; black squares, n=6). Two-way repeated measures ANOVA (group, time) with Holm-Sidak's multiple comparisons test. * $p<0.05$ for effect of treatment at each timepoint below line. Data from historical controls (no DH; grey circles, n=6) displayed to provide physiological context for the ovine model, however no statistical comparisons with these historical controls were made.

Table 1 – Calculations for derived measures

Measure	Calculation
PVR	(PAP – LAP) / PBF
DLC	V_T / (PIP – PEEP)
$AaDO_2$	(FiO_2 x (P_{atm} - P_{H_2O}) – $PaCO_2$ / 0.8) – PaO_2
CaO_2[46]	1.39 x Hb x SaO_2/100 + 0.003 x PaO_2
DO_2[46]	carotid arterial blood flow x CaO_2

PVR = pulmonary vascular resistance; PAP = pulmonary arterial pressure (mmHg); LAP = left atrial pressure, assumed to equal 9 mmHg based on previous studies[47]; PBF = pulmonary blood flow (mL/min); DLC = dynamic lung compliance, V_T = tidal volume (mL); PIP = peak inspiratory pressure (cmH_2O); PEEP = positive end expiratory pressure (cmH_2O); $AaDO_2$ = alveolar-arterial difference in oxygen tension; FiO_2 = fraction of inspired oxygen (%); P_{atm} = atmospheric pressure (760 mmHg); P_{H_2O} = water vapour pressure (47 mmHg); $PaCO_2$ = partial pressure of arterial carbon dioxide (mmHg); PaO_2 = partial pressure of arterial oxygen (mmHg); CaO_2 arterial oxygen content; Hb = arterial haemoglobin concentration (g/dL); SaO_2 = arterial oxygen saturation; DO_2 = cerebral oxygen delivery (mL/min/kg).



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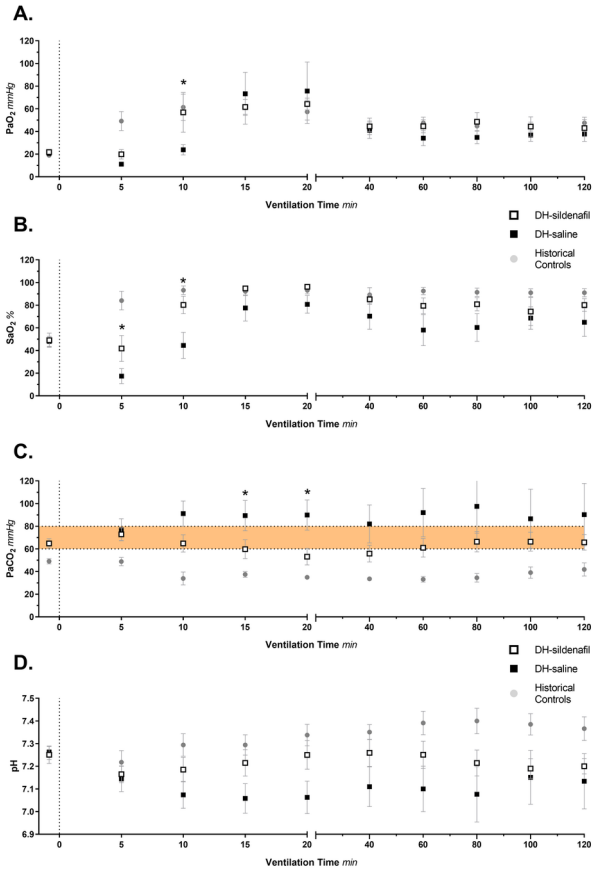

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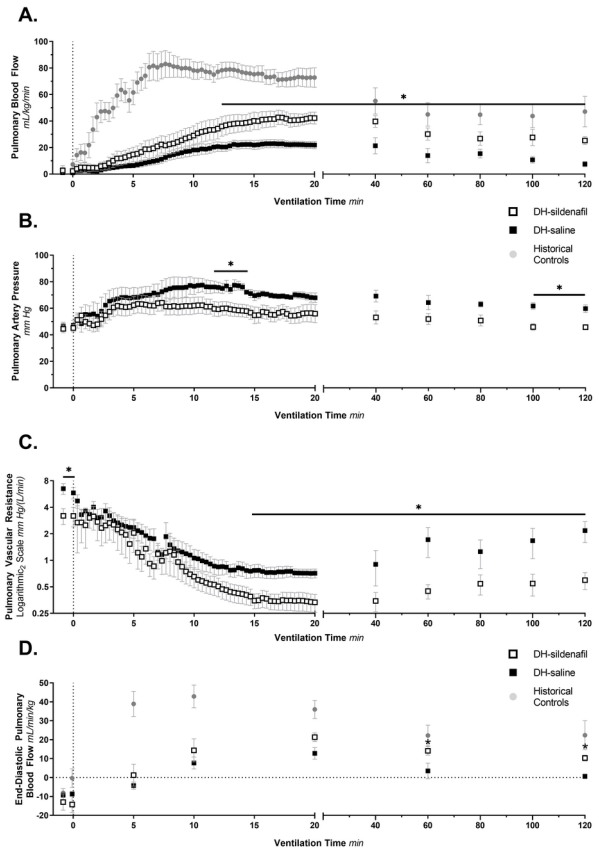

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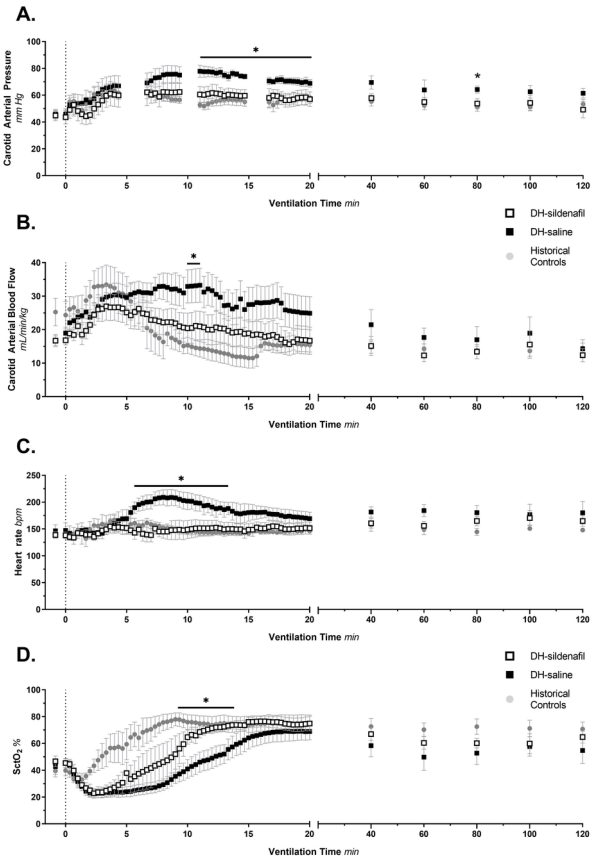

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Title:
Antenatal sildenafil treatment improves neonatal pulmonary hemodynamics and gas exchange in lambs with diaphragmatic hernia

Date:
2019-10-01

Citation:
Kashyap, A. J., Dekoninck, P. L. J., Rodgers, K. A., Thio, M., Mcgillick, E. V., Amberg, B. J., Skinner, S. M., Moxham, A. M., Russo, F. M., Deprest, J. A., Hooper, S. B., Crossley, K. J. & Hodges, R. J. (2019). Antenatal sildenafil treatment improves neonatal pulmonary hemodynamics and gas exchange in lambs with diaphragmatic hernia. ULTRASOUND IN OBSTETRICS & GYNECOLOGY, 54 (4), pp.506-516. https://doi.org/10.1002/uog.20415.