Pulmonary vascular effects of sildenafil on the development of chronic pulmonary hypertension in the ovine fetus

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Persistent pulmonary hypertension of the newborn (PPHN) is a clinical syndrome that is associated with diverse neonatal cardiopulmonary diseases, including birth asphyxia, sepsis, meconium aspiration, and respiratory distress syndrome, or can be idiopathic (15). Physiologically, PPHN results from the failure of the pulmonary circulation to dilate at birth. This syndrome is characterized by sustained elevation of PVR (15), causing extrapulmonary right-to-left shunting of blood across the DA and foramen ovale and severe hypoxemia (15). PPHN is also usually associated with low systemic pressure and low cardiac output because of increased right ventricular afterload and myocardial dysfunction (5, 6). PPHN contributes to significant morbidity and mortality, indicating a need for further study of its pathogenesis and management (35).

An experimental model of chronic intrauterine pulmonary hypertension can be obtained by partial compression or ligation of the DA in the late-gestation ovine fetus (1, 20). In this model, chronic DA compression causes progressive and sustained intrauterine pulmonary hypertension without increased pulmonary blood flow or hypoxemia (1, 20). Past studies have also demonstrated blunted vasodilation to physiological stimuli such as increased PaO2 and shear stress (9, 18, 26, 29, 33) and increased smooth muscle thickness in small pulmonary arteries (16). After delivery, PVR remains elevated despite mechanical ventilation with 100% O2 (1, 20). Mechanisms by which chronic antenatal pulmonary hypertension alters pulmonary vasoreactivity and contributes to the failure of PVR to fall at birth are incompletely understood.

Cyclic guanosine monophosphate (cGMP) is a potent pulmonary vasodilator (12). Hydrolysis of cGMP is achieved predominantly by cGMP-specific phosphodiesterase-5 (PDE-5). PDE-5 is abundantly expressed in lung tissue (21). Preliminary biochemical studies reported that lung PDE-5 activity is markedly elevated during fetal life and then rapidly falls after birth (12). The concomitant decrease in lung PDE-5 activity and in PVR in early transition suggests that PDE-5 activity may be involved in the circulatory adaptation at birth (12). PDE-5 inhibition was also found to cause potent pulmonary vasodilation in late-gestation ovine fetus, suggesting an important role of PDE-5 activity in regulating basal pulmonary vascular tone (39). Moreover, lung PDE-5 activity is increased in experimental conditions with elevated PVR caused by chronic hypoxia (9) or by antenatal closure of the DA (13, 32). Together, these data indicate that PDE-5 may be an important regulator of perinatal pulmonary circulation. However, the

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pulmonary vascular effects of PDE-5 inhibition during the development of chronic pulmonary hypertension are presently unknown.

We therefore hypothesized that PDE-5 inhibition may protect from the consequences of chronic pulmonary hypertension, i.e., from altered vasoreactivity and from vascular remodeling in the perinatal lung. To test this hypothesis, we investigated the pulmonary vascular effects of sildenafil, a potent and selective PDE-5 inhibitor, in chronically prepared late-gestation fetal lambs with chronic pulmonary hypertension obtained by antenatal partial compression of the DA.

METHODS

Animal Preparation

All animal procedures and protocols used in this study were reviewed and approved by the French Ministère de l’Agriculture, de la Pêche et de l’Alimentation before the studies were conducted. Ten mixed-breed pregnant ewes between 130 and 133 days gestation (term = 147 days) were fasted for 48 h before surgery. Ewes were sedated with intravenous pentobarbital sodium (total dose: 2–4 g) and anesthetized with 1% bupivacaine hydrochloride (4 mg) by lumbar puncture. Under sterile conditions, the fetal lamb’s left forelimb was delivered through a uterine incision. A skin incision was made after local infiltration with lidocaine. Polyvinyl catheters (20 gauge) were advanced into the aorta and the vena cava through the axillary vessels. A left thoracotomy exposed the heart and great vessels. Catheters were inserted into the left pulmonary artery (LPA) (22 gauge), main pulmonary artery (20 gauge), and left atrium (20 gauge). An ultrasonic flow transducer, size 6 (Transonic Systems, Ithaca, NY), was placed around the LPA to measure blood flow. An inflatable vascular occluder (OC 10 mm; In Vivo Metric, Healdsburg, CA) was placed loosely around the DA after gentle dissection of adherent connective tissue. The uteroplacental circulation was kept intact, and the fetus was gently replaced in the uterus. An additional catheter was placed in the amniotic cavity to measure pressure. Ampicillin (500 mg) was added to the amniotic cavity before closure of the hysterotomy. The blood flow signals (at least 30 cardiac cycles), with zero blood flow defined as the measured flow value immediately before the beginning of systole (4). Main pulmonary artery, aortic, left atrial, and amniotic catheters were connected to blood pressure transducer (Merlin monitor, Hewlett-Packard). The pressure and flow signals were continuously recorded and processed on a computer (Pentium III 450 Mz) using an analog-to-digital converter system (Lab-View, National Instrument). Data were sampled at a rate of 50 samples/s. Pressures were referenced to the amniotic cavity pressure. Calibration of the pressure transducers was performed with a mercury column manometer. Heart rate (HR) was determined from the phasic pulmonary blood flow signal. PVR in the left lung was calculated as the difference between mean pulmonary artery pressure (PAP) and left atrial pressures (LAP) divided by mean left pulmonary blood flow. Blood samples from the main pulmonary artery catheter were used for blood gas analysis (OSM 3 hemoximeter and ABL 520; Radiometer, Copenhagen, Denmark) and for plasma sildenafil concentration measurements. Plasma sildenafil concentration was measured by using liquid chromatography-tandem mass spectrometry method on silica column with aqueous-organic mobile phase (11).

Drug Preparation

A solution of sildenafil at a concentration of 1 mg/ml (Pfizer, Sandwich, UK) was used.

Experimental Design

Fetal lambs were randomly assigned to one of the following groups: 1) control group (n = 5) infused with saline at a rate of 1 ml/h or 2) sildenafil group (n = 5) infused with sildenafil at a rate of 1 ml/h. This dose was selected from previous studies that have demonstrated substantial effects of sildenafil with oral doses ranging from 100 µg·kg⁻¹·day⁻¹ to 25 mg·kg⁻¹·day⁻¹ in rats and with an oral dose of 150 mg/day in adult patients with pulmonary arterial hypertension (19, 23, 38).

Sildenafil was infused into the venous catheter. Infusion started 6 h after fetal surgery. We induced chronic pulmonary hypertension 24 h after the surgery by inflating the vascular occluder by slowly infusing a small volume of saline into the cuff around the DA, causing a partial compression of the DA (11). The degree of inflation of the occluder was set to increase mean PAP by 15 mmHg from its baseline value. Several readjustments of the occluder inflation were required during the first 2 h of DA compression. Then, continuous inflation pressure was applied in the occluder. Briefly, a 10-ml saline syringe was connected to the occluder. The piston was continuously pressed by a spring whose length was set to obtain the target PAP. Mean PAP and mean aortic pressure (AoP) were measured daily to readjust the pressure in the occluder, if necessary. The DA was compressed for 8 completed days (study period = 192 h).

Four different experimental protocols were included in this study:

Protocol 1: effects of sildenafil on the change in basal pulmonary vascular tone during chronic antenatal pulmonary hypertension. The purpose of this protocol was to determine whether sildenafil would attenuate the progressive rise in pulmonary vascular tone caused by chronic partial compression of the DA. The hemodynamic parameters were measured before and after the beginning of DA compression (at day 0, day 2, day 4, day 6, and day 8). Mean PAP, mean AoP, LAP, amniotic pressure, LPA blood flow, and HR were recorded at 5-min intervals for 30 min and averaged. If required because of a slight drift from the target PAP, readjustment of the inflation pressure of the occluder was performed just after the recordings of the hemodynamic parameters. Daily blood gas analysis was performed. Plasma sildenafil concentrations were measured 24 h and 4 and 8 days after the start of sildenafil infusion. Data obtained in the control group and in the sildenafil group were compared.

Protocol 2: effects of sildenafil on the hemodynamic response to partial DA compression during chronic antenatal pulmonary hypertension. The purpose of this protocol was to determine whether sildenafil would preserve the pulmonary vasodilator response to acute DA compression during chronic pulmonary hypertension. After 96 h of DA compression (at day 4), the vascular occluder was deflated for 20 min and then was reinflated to study the pulmonary vascular response to DA compression. The degree of inflation was set to increase mean PAP by 15 mmHg from its baseline value. DA compression was maintained, and mean PAP was kept constant by readjustment of the degree of inflation of the occluder, as needed. Mean PAP, LAP, mean AoP, amniotic pressure, and left pulmonary blood flow were recorded at 5-min intervals for 70 min. Arterial blood gas tensions and pH were measured during baseline period and during the compression period. Data obtained in control and sildenafil groups were compared.
Protocol 3: effects of sildenafil on the hemodynamic response to increase in fetal PaO₂, during chronic antenatal pulmonary hypertension. The purpose of this protocol was to determine whether sildenafil would preserve the pulmonary vasodilator response to increase in fetal PaO₂ during chronic pulmonary hypertension. After 140 h of DA compression (day 0), fetal PaO₂ was increased by providing 12 l/min O₂ to the ewe through a nasal cannula for 30 min. Hemodynamic measurements (mean PAP, LAP, AoP, pulmonary flow, PVR) were recorded at 5-min intervals starting 30 min before O₂ inhalation, during O₂ inhalation, and for 30 min after O₂ discontinuation. Fetal blood gas tensions and pH were measured at baseline and 25 min after the beginning of O₂ inhalation. Data obtained in control and sildenafil groups were compared.

Protocol 4: effects of sildenafil on pulmonary vascular remodeling and on the development of right ventricular hypertrophy after chronic DA compression. The fetuses were killed with a large dose of pentobarbital sodium after 8 days of DA compression. Fetal weight was recorded. The heart and lungs were rapidly removed in bloc and on the development of right ventricular hypertrophy after 8 days of DA compression, the free wall of the right ventricle weight (10.2 ± 1.5 g, control vs. sildenafil), or in the sildenafil group during O₂ inhalation (P < 0.05, Fig. 1). The gradient between AoP and PAP was similar in the two groups during the study period. Values for mean LAP (2 ± 1 mmHg), HR, arterial blood gas, and pH were not different between study groups at baseline and at day 4 and day 8 of the DA compression period (Table 1). Mean plasma sildenafil concentrations 24 h, 4 days, and 8 days after start of sildenafil infusion were 24 ± 8, 15 ± 6, and 15 ± 3 ng/ml, respectively.

Protocol 2: Effects of Sildenafil on the Hemodynamic Response to Partial DA Compression During Chronic Antenatal Pulmonary Hypertension

In the control group, PAP increased during DA compression from 58 ± 5 to 71 ± 6 mmHg (P < 0.01) (Fig. 2). DA compression did not increase mean left pulmonary blood flow (P < 0.05) (Fig. 2). Mean PVR increased by 25% after DA compression (from 1 ± 0.06 to 1.24 ± 0.06 mmHg·ml⁻¹·min⁻¹, P < 0.05) (Fig. 2). In the sildenafil group, DA compression increased mean PAP from 55 ± 5 to 70 ± 4 mmHg (P < 0.01). Left pulmonary blood flow increased by 80% (from 64 ± 19 to 114 ± 11 ml/min), and mean PVR decreased by 35% (from 0.8 ± 0.1 to 0.5 ± 0.1 mmHg·ml⁻¹·min⁻¹) after DA compression (P < 0.01) (Fig. 2). Mean LAP, mean AoP, and arterial blood gas parameters did not change after the compression period and were not different between groups.

Protocol 3: Effects of Sildenafil on the Hemodynamic Response to Increase in Fetal PaO₂, During Chronic Antenatal Pulmonary Hypertension

Fetal PaO₂ increased from 16 ± 2 to 23 ± 4 mmHg in the control group and from 15 ± 2 to 25 ± 5 mmHg in the sildenafil group during O₂ inhalation (P < 0.05). In both groups, mean PAP did not change during O₂ test (Fig. 3). In the control group, increase in fetal PaO₂ did not change significantly mean left pulmonary blood flow and mean PVR (Fig. 3). In the sildenafil group, mean left pulmonary blood flow increased by 90% (from 91 ± 30 to 171 ± 32 ml/min, P < 0.05), and mean PVR decreased by 60% (from 0.78 ± 0.2 to 0.35 ± 0.06 mmHg·ml⁻¹·min⁻¹, P < 0.05) during the O₂ test (Fig. 3). Mean LAP, mean AoP, and arterial blood gas parameters did not change during the O₂ test and were not different between groups.

Protocol 4: Effects of Sildenafil on Pulmonary Vascular Remodeling and on the Development of Right Ventricular Hypertrophy After Chronic DA Compression

The mean external diameters of the pulmonary vessels were 31 ± 1.6 and 31 ± 1.5 μm in the control and the sildenafil groups, respectively. The degree of muscularization of small pulmonary arteries, as assessed by measurements of percent wall thickness, were similar in both groups (53 ± 2.8 vs. 56 ± 4.5%, control vs. sildenafil). No difference between groups were found in right ventricle weight (10.2 ± 1.2 vs. 9.5 ± 1.5 g, control vs. sildenafil), in left ventricular plus septal weight (13.1 ± 1.8 vs. 13 ± 1.5 g, control vs. sildenafil), or in...
the right ventricle-to-left ventricle plus septum ratio (78 ± 11% vs. 73 ± 9%, control vs. sildenafil).

DISCUSSION

Antenatal DA compression causes progressive increase in PVR, alters pulmonary vasoreactivity, and induces vascular and cardiac remodeling. In this in vivo experimental study, we examined whether these changes in lung circulation can be prevented by selective PDE-5 inhibition. We found that sildenafil attenuated the progressive elevation of basal pulmonary vascular tone observed during chronic DA compression-induced pulmonary hypertension. Furthermore, we found that sildenafil preserved the pulmonary vasodilator response to increase in shear stress and to increase in fetal Po2. However, pulmonary vascular remodeling and cardiac hypertrophy usually observed in this model were not altered by sildenafil. These data suggest that specific inhibition of PDE-5 may prevent from the development of chronic pulmonary hypertension and its consequences on the pulmonary vascular reactivity.
This is the first study to demonstrate a protective effect of prolonged administration of PDE-5 inhibitor on the perinatal pulmonary circulation in an experimental model of chronic pulmonary hypertension. In newborn piglets, brief intravenous sildenafil reversed the acute increase in PVR induced by meconium aspiration syndrome (27). In newborn lambs with PPHN, infusion for 2 h of E4021, a specific PDE-5 inhibitor, caused a significant pulmonary vasodilation (10). However, little is known about the effects of prolonged sildenafil administration on vascular functional and structural changes induced by chronic pulmonary hypertension. Previous investigations showed that sildenafil may attenuate the development of hypoxia- or monocrotaline-induced pulmonary hypertension in adult rats (23, 24). We found that such an effect of sildenafil exists also during the perinatal life. Despite exposure to similar PAP in utero, sildenafil prevented from the progressive rise in basal pulmonary vascular tone observed during chronic intrauterine DA compression in fetal lamb. Pulmonary hypertension is usually associated with abnormal pulmonary vasoreactivity. In the fetal lamb, chronic antenatal DA compression results in the loss of pulmonary vasodilatation to various stimuli (9, 18, 26, 29, 33). In particular, the pulmonary vascular response to increased O2 tension is abolished in this model (9). Furthermore, the vascular response to change in hemodynamic forces is also impaired (29). In the normal fetal lamb, acute compression of the DA abruptly increases blood flow and pressure and causes acute pulmonary vasodilatation through the shear stress-induced release of NO (2). Conversely, chronic pulmonary hypertension in utero impairs flow-induced vasodilation and augments a potent myogenic response, causing a paradoxical vasoconstriction to acute DA compression (29). This abnormal vasoreactivity was observed in our control group, as PVR increased during DA compression after 6 days of pulmonary hypertension. To our knowledge, the effects of PDE-5 inhibition on the pulmonary vascular reactivity have not been yet evaluated in chronic pulmonary hypertension. Our data clearly show that, despite prolonged exposure to high PAP, both increased O2 tension and shear stress induced a decrease in PVR in the sildenafil-treated animals. These results suggest that sildenafil may preserve, at least in part, the physiological pulmonary vascular reactivity in chronic pulmonary hypertension. In addition to functional vascular changes, chronic pulmonary hypertension increases muscularization of small pulmonary arteries and causes right ventricular hypertrophy. Recent studies showed that sildenafil may attenuate vascular remodeling and right ventricular hypertrophy in adult rats with hypoxia- or monocrotaline-induced pulmonary hypertension (23, 24, 38). In our model, the degree of muscularization of small pulmonary vessels and the severity of right ventricular hypertrophy were not altered by sildenafil. The reasons for the discrepancy with previous studies are presently uncertain. Hemodynamic forces such as increased transmural pressure and stretch stress exerts a direct proliferative effect on pulmonary artery smooth muscle cells and contribute to the remodeling process (14, 36). In experimental models with hypoxia- or monocrotaline-induced pulmonary hypertension, vascular remodeling is, at least in part, triggered by the mechanical increase in vascular stretch stress (23, 24, 38). In these models, sildenafil attenuated the rise in PAP (24, 38). Thus the protective effects of sildenafil on the pulmonary vascular remodeling may result from sildenafil-induced drop in PAP. In our model, PAP was kept constant within the study period by readjusting the degree of inflation of the DA occluder. We suggest that, despite sildenafil, the pulmonary vascular bed and the right ventricle were exposed to high stretch stress, leading to significant vascular remodeling and right ventricular hypertrophy.

Surprisingly, pulmonary vascular tone was similar in the control and in the sildenafil-treated fetal lambs at the study baseline (18 h after starting saline or sildenafil infusion). Dipyridamole, a nonspecific PDE-5 inhibitor, was found to cause a brief pulmonary vasodilatation in late-gestation ovine fetuses (39). However, the effects of prolonged PDE-5 inhibition have not been investigated in the fetus. In our study, we did not evaluate the immediate pulmonary vascular response to sildenafil. The mechanisms explaining the lack of late effects of prolonged sildenafil infusion on the basal pulmonary vascular tone are presently unknown. The hypothesis of an inadequate dose of sildenafil is unlikely. Sildenafil inhibits PDE-5 activity by 50% at a concentration of 3.5 nmol/l (34). In the treated animals, we found that sildenafil plasma concentrations ranged from 15 to 25 ng/ml, i.e., from 22 to 38 nmol/l. Alternatively, it may be hypothesized that autoregulatory mechanisms may have prevent from sildenafil-mediated pulmonary vasodilation, including decreased ability to sustain production or effectiveness of endogenous vasodilator, or enhanced production of vasoconstrictor mediators.

Mechanisms that modulate smooth muscle cGMP level are critical in determining basal vascular tone and reactivity. PDE-5 regulates intracellular levels of cGMP by hydrolyzing cGMP to GMP. Thus PDE-5 limit the vasodilator effects of cGMP-mediated vasoactive factors, such as NO and natriuretic peptides on the pulmonary vasculature. A large amount of

### Table 1. Blood gas, HR, and mean AoP, before DA compression (day 0) and after 4 days (day 4) and 8 days (day 8) of DA compression in the control and sildenafil groups

<table>
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<th>Just Before DA Compression</th>
<th>After 4 Days of DA Compression</th>
<th>After 8 Days of DA Compression</th>
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<td></td>
<td>Control</td>
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<td>n</td>
<td>5</td>
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<tr>
<td>pH</td>
<td>7.37±0.04</td>
<td>7.36±0.04</td>
<td>7.37±0.02</td>
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<tr>
<td>PaCO2, mmHg</td>
<td>44±2</td>
<td>49±3</td>
<td>52±5</td>
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<td>PaO2, mmHg</td>
<td>21±5</td>
<td>17±6</td>
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<td>HCO3, mEq/l</td>
<td>25±5</td>
<td>27±2</td>
<td>29±2</td>
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<tr>
<td>BE</td>
<td>0.3±5</td>
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<tr>
<td>HR, beats/min</td>
<td>164±11</td>
<td>170±30</td>
<td>155±9</td>
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<td>AoP, mmHg</td>
<td>45±4</td>
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Means ± SD. BE, base excess, HR, heart rate (beats/min), AoP, aortic pressure (mmHg), DA, ductus arteriosus. P < 0.05.
PDE-5 activity was found in the lung, especially in the human pulmonary artery (21). PDE-5 expression, protein, and activity are elevated in fetus and decrease at birth, indicating that this enzyme may be of particular importance in the pulmonary vascular transition at birth (12). Several investigators have suggested that an increase in PDE-5 activity contribute to the pathophysiology of pulmonary hypertension (17, 23, 24, 38).

Our results support this hypothesis, as sildenafil prevented the functional consequences of pulmonary hypertension. We did not measure the biochemical activity of PDE-5 in the pulmonary vessels. However, in a previous study, Hanson et al. (13) found that lung PDE-5 activity increased by 150% in fetal lambs with DA ligation for 8 days compared with control lambs. Immunocytochemistry study demonstrated that PDE-5 activity was localized to lung vascular smooth muscle (13). We propose that the increase in PDE-5 activity with pulmonary hypertension promote the progressive rise in basal pulmonary vascular tone after DA compression by decreasing cGMP concentrations in vascular smooth muscle cells. In the fetus, both O₂ and shear stress induce pulmonary vasodilation through activation of the NO-cGMP cascade (9, 25, 28). Although shear stress can also stimulate prostacyclin production, prostacyclin-induced pulmonary vasodilation is largely mediated by NO release in the late-gestation fetus (37). We suggest that the loss of pulmonary reactivity to increased O₂ and shear stress in fetus with chronic DA compression may result from an increase in PDE-5 activity. High PDE-5 activity in chronic pulmonary hypertension may dampen the effect of O₂, or shear stress-induced release of NO.

The study itself has some limitations: 1) sildenafil was started 18 h before DA compression and was infused throughout the whole study period. Thus we found that chronic sildenafil treatment improves pulmonary hemodynamics in perinatal pulmonary hypertension when used prophylactically. Whether or not initiation of treatment when pulmonary hypertension has developed may reverse their hemodynamic consequences is presently unknown. 2) Pulmonary blood flow was higher in the sildenafil group than in the control group. Blood flow may alter the hemodynamic forces exerted on the vascular bed.

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wall, such as shear stress (1, 2, 28). Shear stress has been shown to elicit a wide range of physiological vascular responses including K⁺ channel activity and modulation of the NO pathway (30). From our data, we cannot rule out the hypothesis that some effects of the sildenafil on the pulmonary vascular tone or pulmonary reactivity may be related to the sildenafil-induced increase in pulmonary blood flow in our model. 3) In this study, we kept PAP constant throughout the study period by adjusting the degree of the DA compression. The DA narrowing required to obtain the target PAP may have been different in the sildenafil-treated fetuses compared with the controls. In several studies, chronic pulmonary hypertension in utero was obtained by partial or complete fixed ligation of the DA with umbilical tape (13, 33), instead of with an inflatable vascular occluder. However, in such experimental model, PAP is not fully controlled and may vary with PVR. 4) Percent wall thickness of lung vessels from ovine fetuses with DA ligation reached 75% in a previous study (1). In our model, percent wall thickness of lung vessels was found close to 55%. However, lack of homogeneity in animal models and in vascular morphometric techniques may explain the discrepancies between the studies. For instance, duration of the DA compression-induced pulmonary hypertension markedly influences the change in pulmonary vascular wall thickness. Percent wall thickness was reported <50% after 3–6 days of DA compression (1). Complete versus partial compression of the DA may also explain differences in the results between studies. Furthermore, despite attempts to standardize the technique, many factors may alter the measurements of vascular structures, including the technique used to prepare lung samples (level of pressure to perfused the lung vessels, staining of the samples) or diameter of the studied vessels.

In conclusion, our study shows that sildenafil prevents the rise in pulmonary vascular resistance and preserved O₂ and shear stress-induced pulmonary vasodilation in ovine fetus with DA compression. Sildenafil exerts this protective effect without decreasing the development of pulmonary vascular remodeling and right ventricular hypertrophy. We speculate that elevation of PDE-5 activity is involved in the development of pulmonary hypertension and in the altered vasoreactivity in perinatal pulmonary hypertension. We further speculate that chronic sildenafil treatment may have a therapeutic role in perinatal pulmonary hypertension.

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