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Original Article

The pulmonary vasculature in a neonatal porcine model with increased pulmonary blood flow and pressure

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Abstract *Introduction:* Hypertension and hyperperfusion of the pulmonary vascular bed in the setting of congenital cardiac malformations may lead to progressive pulmonary vascular disease. To improve the understanding of the basic mechanisms of this disease, there is a need for clinically relevant animal models which reflect the disease process. *Material and Results:* We randomly allocated 45 newborn pigs, at the age of 48 hrs, to groups in which there was either construction of a 3 mm central aorto-pulmonary shunt, undertaken in 9, or ligation of the left pulmonary artery, achieved in 13. Controls included sham operations in 13, or no operations in 10 pigs. Follow-up was continued for three months. The interventions were compatible with survival in most pigs. The shunts resulted in an acute 85% increase in systolic pulmonary arterial pressure, and a more than twofold increase in pulmonary blood flow. By three months of age, nearly all shunts had closed spontaneously, and haemodynamics were normal. Ligation of the left pulmonary artery resulted in a normal total pulmonary blood flow, despite only the right lung being perfused, and a 33% increase in systolic pulmonary arterial pressure. These haemodynamic changes were maintained throughout the period of study. In both groups, histomorphometry revealed markedly increased muscularity of the intra-acinar pulmonary arteries. Circulating levels of endothelin were normal in the shunted animals, and elevated in those with ligation of the left pulmonary artery. *Conclusion:* In neonatal porcine models of pulmonary vascular disease, created by construction of 3 mm central aorto-pulmonary shunts and ligation of one pulmonary artery, we observed histopathological changes of the pulmonary vasculature similar to early hypertensive pulmonary vascular disease in humans. Elevated circulating levels of endothelin were associated with abnormal haemodynamics rather than abnormal pathology. These findings could be valuable for future studies on the pathogenesis of hypertensive pulmonary vascular disease associated with congenital cardiac malformations.

Keywords: Hypertensive pulmonary vascular disease; pulmonary hypertension; congenital heart disease; endothelin

HYPERPERFUSION OF THE PULMONARY VASCULAR bed in the setting of congenital cardiac malformations may lead to progressive hypertensive pulmonary vascular disease. Although nowadays it is rare to see the development of advanced hypertensive pulmonary vascular disease, abnormal pulmonary vascular reactivity is still a major contributor to morbidity and mortality after surgical correction. Accumulating evidence now documents marked alterations in basic vascular biology in the diseased state.[1–3] In particular, injury and dysfunction to the endothelial cell are suggested as key early events in hypertensive pulmonary vascular disease. If we are to be able to study possible pathogenic mechanisms in relation to changes in vascular biology, it is essential to develop clinically relevant

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animal models which reflect the disease processes. The aim of the present study, therefore, was to establish neonatal porcine models of hypertensive pulmonary vascular disease. High-pressure hyperperfusion was induced on the one hand by creating a central aorto-pulmonary shunt, and on the other hand by ligation of the left pulmonary artery. Pulmonary haemodynamics and pulmonary vascular morphology were studied, and the circulating levels of endothelin and von Willebrand factor were measured.

Materials and methods

Experimental design

The experiments were performed after approval from The Danish Department of Justice Ethical Committee on Animal Experimentation. We allocated randomly 45 newborn mixed Danish Landrace and Yorkshire pigs at the age of 48 hours, 23 of the cohort being female, and with the overall group weighing 1824 ± 288 grams, with a range from 1150 to 2600 grams, to have either construction of a central aorto-pulmonary shunt, achieved in 9 animals, or ligation of the left pulmonary artery, undertaken in 13 animals. The remaining piglets formed the control groups, with 13 undergoing a sham operation and 10 remaining without surgery. Three months later, the animals underwent cardiac catheterisation and were then sacrificed during continued anaesthesia.

Anaesthesia and monitoring. The animals were premedicated with azaperonum (8 mg/kg) and midazolam (0.2 mg/kg). Etomidate (400 mg/kg) was given optionally to facilitate endotracheal intubation. Anaesthesia was maintained with halothane (0.25–1.50%) using a Servo 900 ventilator. Ampicillin (50 mg) was given before and after surgery. During surgery and cardiac catheterisation, the electrocardiogram, central temperature, arterial blood pressure, central venous pressure and peripheral oxygen saturation were monitored continuously.

Postoperative care and follow-up. Dobutamine (4–16 µg/kg/min) was given for the first 2–4 postoperative hours. The animals could be weaned off the ventilator after 6–10 hours. Buprenorphine (0.03 mg/kg) was given immediately postoperatively and repeated every 12 hours as required. Ampicillin (50 mg/day) was continued for the first week. During surgery and until able to resume bottle feeding, a mixture of glucose (41.6 gr/l), sodium (1.2 gr/l) and potassium chloride(1.5 gr/l) was given iv. (6–8 ml/kg/hr). Nutrition was continued every four hour by bottle. After three weeks, the animals could be weaned off the bottle. Elemental iron (100 mg/kg) was given at one week of age. Animals were weighed daily during the first two weeks and subsequently weekly. During the first two weeks, patency of the shunts was assessed daily by auscultation, after which time it had to be abandoned due to lack of cooperation from the animals.

Surgical protocol

After sterile preparation, a left anterior thoracotomy was performed through the fourth intercostal space. After interventions, the pericardium was left open and the chest closed in layers.

Pilot study

The shunt model. To induce as much flow and pressure on the immature pulmonary circulation as possible, it was necessary to determine the maximal diameter and minimal length of the extracardiac shunt. Shunts with an effective diameter of 3 mm, equal to approximately half of the internal diameter of the aorta and the pulmonary trunk, were found to be compatible with survival in the newborn pig, whereas larger shunts induced acute cardiac failure and death within 24 hours. Additionally, because of the short length of the porcine ascending aorta, it proved difficult to interpose larger shunts. Furthermore, in the newborn pig, it was not possible to achieve conventional tangential clamping because of the small lumen and thick walls of the vessels.

Construction of the central aorto-pulmonary shunt (Fig. 1). Ribbons were placed around the pulmonary trunk, the distal aortic arch, the brachiocephalic trunk and the left subclavian artery to allow total cross clamping. Two types of anastomoses were performed.

End-to-side anastomosis. This was achieved in 6 animals. A 6mm Gore-Tex vascular prosthetic graft was interposed end-to-side to the intact proximal ascending aorta, shortened maximally, adjusted to the pulmonary trunk, and sutured end-to-side to the pulmonary trunk keeping the loops on the anterior side loose. During a short period of cross clamping, the vessels were incised, a 3mm arteriotomy was created using a vascular punch (Deknatel Inc, MA, USA), and the suture line tightened. The punch had been modified (Intermedico Inc. Copenhagen, Denmark) to fit into the vascular graft and to produce a 3mm hole.

Side-to-side anastomosis. In these 3 cases, the intact proximal ascending aorta was sutured directly

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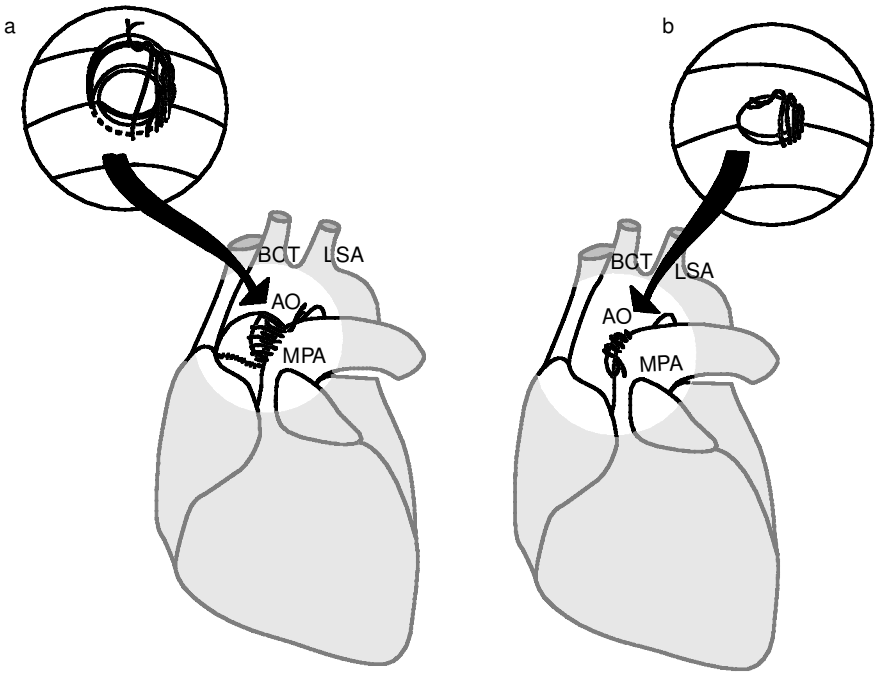

Figure 1.
Diagram of surgical techniques used to create the central aorto-pulmonary shunts. See text for details. End-to-side anastomosis (A), and side-to-side anastomosis (B). Abbreviations: AO: ascending aorta; BCT: brachiocephalic trunk; LSA: left subclavian artery; MPA: main pulmonary artery.

side-to-side to the intact pulmonary trunk. Half the circumference was sutured with interrupted sutures, allowing for potential growth of the anastomosis. The same technique of cross-clamping, incision, punching of the arteries and tightening of the running suture was used.

Ligation of the left pulmonary artery. The pulmonary trunk and the right and left pulmonary arteries were isolated by dissection, and the left pulmonary artery ligated.

Sham operations. After thoracotomy, the central vascular structures were exposed and dissected as if an interventional procedure was to be performed.

Unoperated animals. Newborn pigs, from the same litters as the pigs undergoing surgery, were taken from the sow at two days of age, and housed under the same conditions as the animals undergoing surgery.

Haemodynamic study

Pressures were measured using fluid filled catheters and Uniflow pressure transducers (Baxter Corporation, Uden, The Netherlands), amplified and recorded on a Sirecust 961 multichannel recorder (Siemens, Erlangen, Germany). Systolic, diastolic and mean pressures were recorded at end-expiration with the ventilator turned off. Blood gases were analysed using an ABL510 Haemoximeter (Radiometer Inc., Copenhagen, Denmark).

Acute haemodynamic study. The acute haemodynamic effects were studied invasively with the chest open. The external carotid artery was cannulated with a 20 G arterial cannula and the pulmonary trunk was punctured with a 21 G needle. Pulmonary and systemic pressures were recorded simultaneously and blood gases taken immediately after. Measurements were repeated twice with an interval of 60 sec and the mean value used.

Haemodynamic study after 3 months. For cardiac catheterisation, the neck vessels were exposed through a right cervical incision. Via the carotid artery and the external jugular vein, 6 French multipurpose cardiac catheters (Cordis-Europe, Ooestende, The Netherlands) were inserted. Pressures and blood gases were recorded at the superior and inferior caval veins, the right atrium, the right ventricle, the pulmonary trunk, the right and left pulmonary arteries, the pulmonary capillary wedge, the ascending aorta, and the left ventricle. Measurements were repeated twice at each position and the mean value used. The flow of blood to the lungs, and the ratios of pulmonary to systemic flows, were calculated using the Fick principle.

Structural study

After cardiac catheterisation, a sternotomy was performed and the heart and lungs were freed of adhering pleural membranes. While under deep anaesthesia and continued ventilation, the animals were sacrificed by exsanguination. The trachea was

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clamped distally at end-inspiration, and the heart and lungs removed as a block, permitting gross examination of the pathological sequels of the induced malformations.

Pulmonary vascular histomorphometry. Immediately after death, small blocks of tissue, measuring approximately 15 × 15 × 4 mm, were harvested from the central part of each pulmonary lobe and promptly fixed in neutral buffered formaldehyde. After routine paraffin embedding, 5 µm sections were cut and stained with elastic Orcein and Van Gieson. Each section was systematically examined, and all suitable pulmonary arteries accompanying terminal and respiratory bronchioles and alveolar ducts were measured as described below, blinded to the observer.

From the microscope (Olympus BX50, ocular × 5, lenses × 20 and 40), the picture of each pulmonary artery was digitalised (Data Translation frame grabber) and transferred to a Pentium computer via a camera (Sony Hyper HAD). From the digitalised picture of each vessel, the level of accompanying airway (terminal or respiratory bronchiole, or alveolar duct) was noted and morphometrical analysis performed. The inner margin of the internal elastic lamina was traced manually. From this tracing, the longest axis, and subsequently the perpendicular axis, was found by the software programme (SigmaScan Pro 3.0 (Jandel Scientific Corp., CA, USA)). This procedure was repeated for the inner margin of the external elastic lamina. Subsequently, the thickness and proportional thickness of the medial wall were calculated as

$$\text{media thickness} = \frac{\text{ext. diameter} - \text{int. diameter}}{2}$$

and

$$\text{percentage media thickness} = \frac{\text{media thickness}}{\text{ext. diameter}} \times 100.$$

Assuming that the elliptical form of the vessel was due to an oblique sectioning, we took the measured perpendicular diameter as the true diameter of the vessel.[4]

Biochemical study

Blood samples were taken randomly from 15 newborn pigs before surgery for assessment of endothelin and von Willebrand factor, and from all animals at three months. Bloods were drawn directly from the ascending aorta and the pulmonary trunk into cooled sodium EDTA tubes for endothelin, or citrate tubes for von Willebrand factor, and immediately thereafter centrifuged at 4000 **g** at 4°C for 10 min. The platelet free plasma was snap frozen in liquid nitrogen and subsequently stored at −80°C until analyses.

Endothelin was measured by radio-immuno assay after preliminary acidification and extraction on C18 columns (Nichols Institute Diagnostic, Wychen, The Netherlands). The volume of plasma used for the extraction was 2 ml. The method determines Endothelin-1, 2, and 3, with relative specificities of 100%, 67%, and 84%, respectively. A similar method has previously been used in pigs.[5]

Von Willebrand factor was measured with the in house three layer ELISA technique, using a monoclonal anti-porcine von Willebrand factor antibody (Porton Speywood Wales Inc.).

Statistical analyses

Haemodynamic data are given as mean plus or minus standard deviation, along with the standard error of the estimate. Differences before and after intervention were compared by paired t-test, and the two groups by unpaired t-test. The groups of animals undergoing surgery were compared at follow-up using one way analysis of variance. The post hoc test used was Dunnetts test, permitting multiple comparisons against the unoperated and the sham-operated animals.

Data on pulmonary vascular histomorphometry for medial thickness were logarithmically transformed to emphasise relative changes and those due to lack of uniformity of variance within and between the animals. For each level of accompanying airway, we calculated the thickness of the vessel wall as the geometric mean of medial thickness, taking the 95% confidence intervals of the median thickness. Differences in pulmonary vascular histomorphometry were compared using a mixed model analysis of variance with three components of variance (σ^2): $\sigma^2_{\text{LUNG LOBE}}$ variance within the lung lobe; σ^2_{ANIMAL} variance witin the animal; σ^2_{GROUP}, variance within the group. For each level of the intra-acinar pulmonary arterial pathway, coefficients of variance were calculated as the standard deviation of the logarithmically transformed medial thicknesses. The estimate of the total variance for the individual measurement at each level of accompanying airway was calculated as the total sums of squares,

$$\sigma^2_{\text{TOTAL}} = \sqrt{(\sigma_{\text{LOBE}})^2 + (\sigma_{\text{ANIMAL}})^2 + (\sigma_{\text{GROUP}})^2}\,.$$

Due to the lack of uniform variance of the data relating to endothelin, these were logarithmically transformed, thereby matching the assumptions for normal distribution. These parameters are given as

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the geometric mean, that is the median with 95% confidence intervals, whereas the measurements for von Willebrand factor are given as mean plus or minus the standard deviation of the raw data. The newborns and unoperated controls at three months were compared by unpaired t-test, and those undergoing interventions at follow-up using one way analysis of variance (Dunnett's post hoc test).

The number of animals allocated for construction of a shunt (9) was smaller than the animals allocated for ligation of the left pulmonary artery. It was considered ethically incorrect to continue allocation to this group when it became obvious that continued patency of the shunts was not being achieved. Bearing the subsequent risk of bias in mind, we retained those shunted animals in the statistical analyses. Statistical software used was the SigmaStat 2.0 package (Jandel Corp., CA, USA) for hemodynamic and biochemical data analysis and the SAS 6.12 package (SAS Inc. Cary, NC) for analyses on pulmonary vascular histomorphometry. We considered a p value of smaller than 0.05 to be significant.

Results

All animals survived the surgical procedures, but 12 (26%) died during follow-up; seven on the first postoperative day, two during the first postoperative week, and three later. There were four deaths in each group. Causes of death were cardiac failure in two shunted animals, respiratory distress in five, septicaemia in one, while death was unexplained in four animals. All non-operated animals survived the period of follow-up. Weight at birth was similar in all groups. At three months, body weight and body surface area in the four groups were: controls 30 ± 4 kg and $0.83 \pm 0.08\ m^2$, sham operation 27 ± 7 kg and $0.75 \pm 0.14\ m^2$, aorto-pulmonary shunt 22 ± 7 kg and $0.68 \pm 0.14\ m^2$, and ligation of the left pulmonary artery 29 ± 7 kg and $0.81 \pm 0.13\ m^2$ ($p = 0.16$).

Haemodynamic study

Acute haemodynamic study (Table 1). Significant changes in haemodynamic indexes were found in both high-pressure hyperperfusion models immediately after surgical intervention, whereas no changes were found after sham operation (data not shown).

Aorto-pulmonary shunt: Systolic pulmonary arterial pressure increased by 85%, and the mean pressure by 61%, whereas diastolic pulmonary arterial pressure and systemic arterial pressures did not change significantly. Assuming no change in the mixed venous oxygen saturation, the increase in pulmonary oxygenation reflected a more than twofold increase in pulmonary blood flow.

Ligation of the left pulmonary artery also increased the pressures in the pulmonary trunk significantly, the systolic by 33% and the mean by 17%. The pressure changes approximated half the increases achieved after creation of the shunt ($p = 0.008$ and $p = 0.006$). Pulmonary arterial oxygen saturation was unchanged, reflecting that the total pulmonary blood flow was normal despite only the right lung being perfused.

Table 1. Acute haemodynamic study.

	Shunt (n=9)				Ligation (n=13)			
	Before	After	% Relative difference	p-value	Before	After	% Relative difference	p-value
Pulmonary arterial pressure (mmHg)								
Systolic	31±6	56±13	85±19	<0.001	29±4	39±5	33±6	0.008
Diastolic	9±2	12±4	30±16	0.25	11±4	10±3	−3±5	0.03
Mean	17±3	29±10	61±16	<0.001	17±3	20±2	17±4	0.006
Systemic arterial pressure (mmHg)								
Systolic	61±6	67±10	10±5	0.69	68±8	69±9	1±3	0.14
Diastolic	32±3	30±7	−4±5	0.53	32±5	32±6	2±4	0.68
Mean	43±5	42±7	−1±6	0.66	45±5	44±6	−1±3	0.96
PAP/SAP								
Mean ratio	0.38±0.11	0.68±0.20	67±18	<0.001	0.37±0.06	0.44±0.06	20±4	0.006
Pulmonary arterial O_2 saturation (%)	63±15	86±10	44±14	0.003	65±10	71±8	9±5	0.11
Heart rate (min^{-1})	115±16	144±24	26±7	0.01	122±21	120±23	−1±3	0.57

Values are mean ± SD and relative differences of means ± SEE. Before = before surgical intervention; After = 30 min after surgical intervention; PAP = pulmonary arterial pressure; SAP = systemic arterial pressure. Sham operation (data not shown) did not change haemodynamic indexes

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Table 2. Three months haemodynamic study.

	Shunt (n=5)	Ligation (n=9)	Sham (n=9)	Control (n=10)	p-value
Pulmonary arterial pressure (mmHg)					
Systolic	24±6	32±8	24±3	24±4	0.006
Diastolic	12±4	17±8	12±4	12±3	0.11
Mean	16±4	24±9	16±3	16±3	0.01
PAP/SAP (mean ratio)	0.26±0.12	0.34±0.09	0.25±0.07	0.24±0.06	0.01
Pulmonary capillary wedge (mmHg)	10±2	14±3	13±3	11±2	0.52
Right atrial pressure (mmHg)	6±2	9±3	6±5	7±4	0.06
O_2 saturation (%)					
Pulmonary arteries	70±13	68±11	71±13	76±4	0.34
Aorta	99±1	99±1	99±1	99±1	
Right atrium	73±6	67±11	72±10	77±5	0.43
Qp ($l/min \times m^2$)	4.9±1.5	4.4±2.0	5.9±2.4	5.6±1.4	0.45
Rp ($U \times m^2$)	1.3±0.9	2.7±2.2	0.6±0.2	0.8±0.2	0.025

Values are mean ± SD. PAP = pulmonary arterial pressure; Qp = pulmonary blood flow; Rp = pulmonary vascular resistance; SAP = systemic arterial pressure; $mmHg/l \times min^{-1}$. Se-haemoglobin in the four groups were 5.5 ± 0.7, 5.6 ± 0.5, 5.2 ± 0.6, and 5.8 ± 0.5 mmol/l (p = 0.34). The index of oxygen consumption for pigs at this age was assumed to be 150 ml/min/m^2 and oxygen capacity for haemoglobin 1.34 ml O_2/g. Pulmonary vascular resistance was calculated using Poiseuilles' equation. Body surface area was calculated using Brody's empirical formula for pigs ($0.097 \times BW\ (kg)^{0.633}$) (6)

Haemodynamic study after 3 months (Table 2). **Aorto-pulmonary shunt:** Neither pulmonary nor systemic arterial pressures were different from pressures in the animals not having an operation, or else having a sham operation. The mean ratio of pulmonary to systemic flows was 1.0 ± 0.3, indicating closure of the shunt. The differences in the mean pulmonary blood flow and pulmonary vascular resistance index compared to control groups could be due to chance.

Ligation of the left pulmonary artery: Pulmonary arterial pressures were significantly higher than in the animals undergoing sham operations, or having no operation, the systolic by 33% and the mean by 50%. Systemic arterial pressures were not different, increasing the ratio of mean pulmonary to systemic pressures by 42%. The pulmonary vascular resistance index was also significantly higher compared to both control groups (2.7 ± 2.2 vs. 0.6 ± 0.2 and 0.8 ± 0.2 $U \times m^2$, p = 0.025). A trend towards a lower pulmonary blood flow index was also suggested (4.4 ± 2.0 vs. 5.6 ± 1.4 and 5.9 ± 2.4 $l/min \times m^2$), although values did not reach statistical significance.

Sham operation: Differences in haemodynamic indexes compared to the animals not submitted to operation could all be due to chance.

Structural study

Gross pathology. **Aorto-pulmonary shunt:** Only one shunt was found patent at autopsy. In the remaining animals, the punctured holes had closed by intimal overgrowth, and the inserted Goretex tube grafts had thrombosed.

Left pulmonary arterial ligation: The ligation was complete in all animals. A dense subpleural network of vessels was observed on the surface of the paler left lung of all animals.

Sham operation: Except for left pleural and pericardial adhesions, which were found in all animals, the gross structure was normal.

Pulmonary vascular histomorphometry (Table 3, Figs 2 and 3). **Aorto-pulmonary shunt:** The medial thickness was significantly increased by 46%, 41%, and 46% in arteries accompanying terminal and respiratory bronchioles, and alvelolar ducts, respectively compared to both control groups (p < 0.001). The differences in median medial thickness between the left and the right lungs could be due to chance.

Left pulmonary arterial ligation: *Comparison between right and left lung* – In the right hyperperfused lung, the differences in median medial thickness of pulmonary arteries accompanying terminal and respiratory bronchioles, and alveolar ducts, was significantly increased by 42%, 49%, and 48% compared to the left lung. *Comparison between ligated animals and control groups* – Medial thickness of the vessels in the hyperperfused right lung was significantly increased by 43%, 40%, and 41% in vessels accompanying terminal and respiratory bronchioles, and alveolar ducts, compared to the two control groups. The differences in muscularity of pulmonary

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Table 3. Pulmonary vascular histomorphometry.

Accompanying airway	Surgical group ($n_{animals}/n_{arteries}$)	Medial thickness (μm)		% Relative difference	Coefficients of variance	
		Left lung	Right lung		p-value	$\sigma_{LOBE, ANIMAL, GROUP, TOTAL}$
Terminal bronchioles	Shunt (5/150)	12.9 (10.0–15.5)	13.5 (10.9–16.8)	4.5 (−13.7–22.8)	0.62	$\sigma_{LUNG\ LOBE}$ =30%
	Ligation (9/291)	7.3 (6.4–8.2)	12.5 (11.0–14.3)	54.7 (43.2–66.2)	<0.001	σ_{ANIMAL}=15%
	Sham (8/174)	7.5 (6.4–8.7)	7.2 (6.1–8.5)	−4.2 (−18.2–9.8)	0.55	σ_{GROUP}=15%
	Control (10/244)	7.1 (6.3–8.0)	7.2 (6.3–8.3)	0.8 (−11.4–12.9)	0.90	σ_{TOTAL}=36%
Respiratory bronchioles	Shunt (5/134)	9.7 (8.4–11.2)	9.1 (7.9–10.6)	−6.1 (−20.3–8.1)	0.39	$\sigma_{LUNG\ LOBE}$ =28 %
	Ligation (9/267)	4.7 (4.3–5.2)	9.2 (8.3–10.3)	66.9 (56.7–77.1)	<0.001	σ_{ANIMAL}=11%
	Sham (8/182)	6.2 (5.5–7.0)	5.4 (4.7–6.1)	−14.6 (−26.5–−2.6)	0.02	σ_{GROUP}=11%
	Control (10/334)	5.6 (5.1–6.1)	5.5 (4.9–6.0)	−2.4 (−11.7–6.9)	0.61	σ_{TOTAL}=32%
Alveolar ducts	Shunt (5/74)	6.5 (5.3–8.1)	6.4 (4.9–8.2)	−2.6 (−27.9–22.7)	0.84	$\sigma_{LUNG\ LOBE}$=29%
	Ligation (9/252)	3.0 (2.6–3.5)	5.8 (5.0–6.8)	66.4 (51.6–81.2)	<0.001	σ_{ANIMAL}=21%
	Sham (8/127)	3.8 (3.2–4.6)	3.3 (2.7–4.1)	−14.2 (−34.0–5.6)	0.15	σ_{GROUP}=16%
	Control (10/252)	3.3 (2.9–3.8)	3.5 (3.0–4.1)	4.7 (−9.7–19.1)	0.52	σ_{TOTAL}=39%

Values are median (95% confidence interval) and % relative differences of means (95% confidence interval) of the medial thickness in pulmonary arteries accompanying terminal and respiratory bronchioles and alveolar ducts. Coefficients of variance (σ) are given as: $\sigma_{LUNG\ LOBE}$ = variance within the lung lobe; σ_{ANIMAL} = variance witin the animal; σ_{GROUP} = variance within the group, and σ_{TOTAL} = the total sums of squares. See text (statistical analyses) for details. Due to insufficient preparation and staining of one sham operated pig, only eight animals were included in the measurements

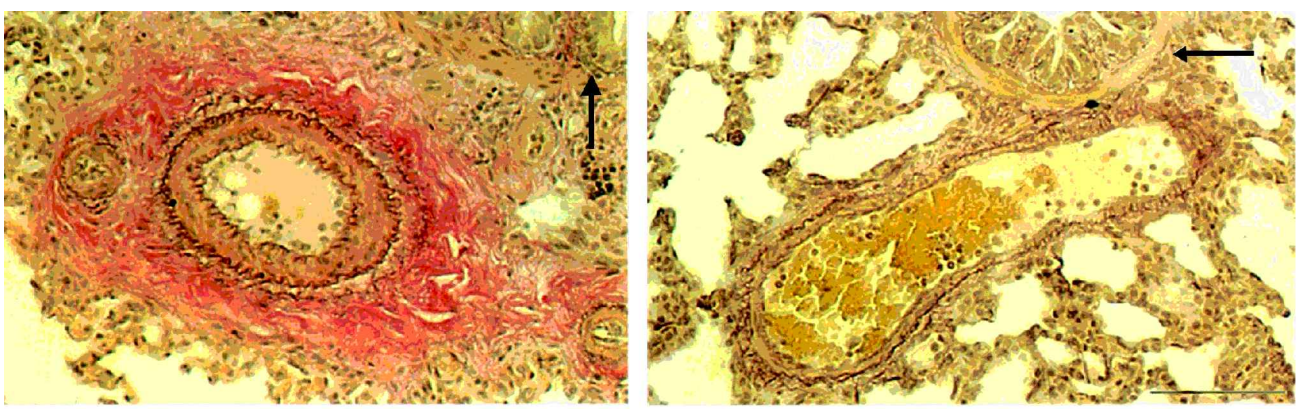

Figure 2.
Photomicrographs of pulmonary arteries and accompanying terminal bronchioles from (a) a shunted pig and (b) an unoperated control pig. In shunted pigs (a), despite normalised haemodynamics, there was markedly increased medial thickness along the entire intra-acinar pulmonary arterial pathway, compared to the unoperated (b) and sham-operated pigs. Elastic Orcein and Van Gieson staining; ——*: 100 μm;* ⟶ *: accompanying terminal bronchioli.*

arteries between the left hypoperfused lung and the left lung of the two control groups could be due to chance.

Sham operations had not changed the overall muscularity of the pulmonary arteries accompanying terminal and respiratory bronchioles and alveolar ducts after three months follow-up compared to the animals in which no surgery had been undertaken. At all three levels of the pulmonary arteries measured, differences in median medial thickness between left and right lung were negative, reaching statistical significance only at the level of arteries accompanying respiratory bronchioles ($p = 0.02$). This could indicate that the sham operation in itself had caused an increase in muscularity, increasing the risk of underestimating minor differences. In an extended mixed model analysis of variance, nonetheless, including all three levels of the pulmonary arterial pathway, the overall relative difference between the left and the right lung in the sham group was −10.4(−22.2–1.4)% ($p = 0.59$).

Biochemical study (Table 4). The temporal or interventional differences in the means between the two

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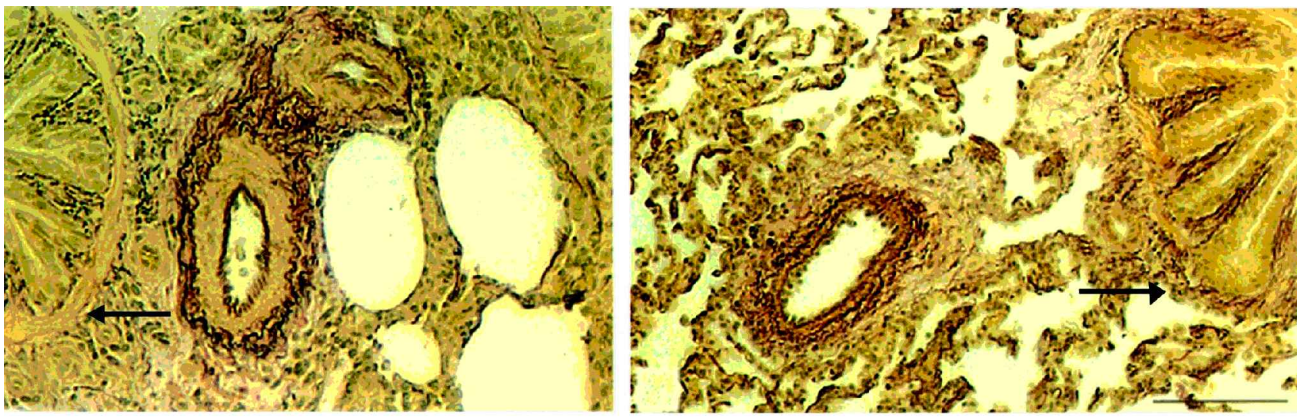

Figure 3.
Photomicrographs of pulmonary arteries and accompanying terminal bronchioles from a pig in which one pulmonary artery was ligated. (a) shows the hyperperfused right lung, with increased muscularity to the same extent as in shunted animals, and (b) the contralateral hypoperfused lung. Elastic Orcein and Van Gieson staining; ——— : 100 μm; ⟶ : accompanying terminal bronchioli.

Table 4. Circulating endothelin and von Willebrand factor.

	Shunt (n=5)	Ligation (n=9)	Sham (n=9)	Control (n=10)	p-value	Newborn (n=15) vs Control	Difference	p-value
Endothelin (pg/ml)								
Aorta	2.4 (1.6–3.7)	4.6 (2.6–8.4)	2.1 (1.4–2.9)	2.6 (1.1–3.5)	0.02	6.2 (3.8–12.1)	0.8 (0.3–1.4)	0.004
Pulmonary arteries	2.6 (1.1–4.2)	5.1 (2.9–8.5)	2.1 (1.6–2.9)	2.5 (1.8–3.4)	0.02	6.1 (3.7–10.0)	0.9 (0.4–1.4)	0.003
von Willebrand factor (arb.u./ml)								
Aorta	1.2±0.4	0.9±0.3	1.1±0.3	0.9±0.3	0.22	0.6±0.2	0.3±0.3	0.08
Pulmonary arteries	0.8±0.3	1.1±0.3	0.9±0.3	0.8±0.3	0.17	0.6±0.2	0.2±0.2	0.21

Values are median (95% confidence interval) for data referring to endothelin, and mean ± SD for those relating to von Willebrand factor. The data concerning endothelin data were logarithmically transformed due to the known heteroscedasticity. arb.u: arbitrary unit

sites of sampling in the ascending aorta and the pulmonary trunk of both endothelin and von Willebrand factor could all be due to chance.

Endothelin: In the newborns, the circulating levels of endothelin were more than two-fold higher compared to both control groups at three months ($p = 0.003/0.004$).

Aorto-pulmonary shunt: The differences in the mean circulating level of endothelin compared to the two control groups could be due to chance.

Left pulmonary arterial ligation: The circulating level of endothelin was significantly higher by 80–90% at three months compared to animals having no or sham operation ($p = 0.02$).

Sham operations had not changed the circulating level of endothelin during the three months period of follow-up.

von Willebrand factor: The differences in the mean values among the groups were not great enough to exclude the possibility that the temporal and interventional differences were due to random sampling variability.

Discussion

We have shown that high-pressure hyperperfusion of the immature pulmonary circulation of the newborn pig can produce significant alterations in the pulmonary vasculature. To our knowledge, this is the first report of a model providing a large left-to-right shunt distal to the tricuspid valve created in the high pulmonary vascular resistance state of the newborn pig. Auscultation showed that the shunts were patent at two weeks of age. By the age of three months, nonetheless, nearly all the shunts had closed spontaneously with intimal overgrowth. Speculating that the fixed size of the shunt could have posed a potential problem for persisting patency, we changed our surgical technique to produce a modified "window". This also produced no

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improvement in patency at three months. Thus, if we are to achieve persistent patency of an aorto-pulmonary shunt in neonatal pigs, a two-stage procedure may be necessary. Yucatan miniature pigs with heritable ventricular septal defects might provide a natural model.[7] These animals, however, usually develop only mild pulmonary vascular changes, indicating that the majority of these defects are relatively small. The majority of experimental models have been created in mature animals.[8–10] Besides the lack of congenital imitation, the reported failure to induce sustained hypertensive pulmonary vascular disease may, in these models, be explained by the mature state of the pulmonary circulation. In contrast, marked haemodynamic and pulmonary vascular changes were achieved in the elegant fetal lamb model described by Reddy et al.,[11] and in a model using newborn calves.[12] The use of larger experimental animals obviously solves a number of technical problems. Porcine models, however, carry the advantage that the development of the pulmonary circulation compares well with that of humans, although occurring at a faster rate.[13]

Unilateral pulmonary arterial ligation was technically simple and resulted in sustained pulmonary hyperperfusion and mild hypertension in the lung fed by the unligated artery during the three months period of study. This is in accordance with one other experimental study,[14] and with clinical observations in some children with congenital absence or severe stenosis of one pulmonary artery.[15]

In the present study, 12 animals (26%) died during follow-up. Most deaths occurred at the beginning of the study. With experience, operating, and hence ventilation, time could be considerably shortened, reducing the incidence of respiratory complications, which were the major causes of death.

By three months of age, a significant increase in muscularity of the pulmonary vasculature was found in the shunted animals, despite normalised pulmonary haemodynamics and closure of nearly all shunts. The pathological changes correspond to the so-called grades A and B in the classification of hypertensive pulmonary vascular disease provided by Rabinowitch and her colleagues in humans.[16] Whether the structural changes would be reversible with time, if the animals were allowed to survive, is not known. Although induced in older animals, similar findings have been reported by Rendas et al.,[17] who suggested that the original effect of a shunt was irreversible. Whether the observed structural changes are of significance, and reflect an abnormal reactivity of the pulmonary vasculature, requires further studies.

Increased muscularity to the same extent as found in the shunted animals was also observed in the hyperperfused and moderately hypertensive right lung of the pigs who had had the left pulmonary artery ligated. This corroborates a previous study by Haworth et al.,[14] and suggest failure of the pulmonary vasculature to adapt and develop normally due to the hyperperfusion and hypertension induced on the immature pulmonary circulation. When establishing this model in pigs one to two weeks of age, Vitvitsky et al.[18] have reported a loss of endothelium-dependent vasodilation, but neither increased vascular muscularity nor haemodynamic changes were found at 12 weeks of age. This suggests that timing of the surgical intervention has significant impact on the subsequent vascular response. Also, medial thickness was assessed in distended vessels after pressure injection, which may affect diseased vessels differently than healthy ones,[19] a methodological difference that could be of importance.

In this study, to standardise the measurements of the medial thickness, the perpendicular diameters of the elastic laminas were used, taking the oblique sectioning of the vessels into account.[4] The same morphometrical method was also used by Rabinowitch et al.[16] In the majority of previous studies however, medial thicknesses have been calculated as the mean of four readings across the diameters, assuming a circular appearance of the vessels.[9,10,14] Data have been presented only as mean plus or minus the standard error, without analysis of variance, which makes comparison with our results difficult. Our analysis of variance showed that measurement error is still to be encountered with standardized measurements, and that a substantial number of measurements, in particular within a lung lobe (coefficients of variance being 30%, 28%, and 29% at the three levels of accompanying airway (Table 3), is necessary to minimise the error.

Increased levels of endothelin have been described in children and adults with established hypertensive pulmonary disease.[20,21] It is not known whether an increased level is a marker or a mediator of the disease. In the fetal lamb model of hyperperfusion and hypertension, Wong et al.[22] found increased levels and altered vasoconstrictor response to endothelin. In our study, despite a quantitatively similar degree of increased pulmonary vascular muscularity, levels of endothelin were normal in the animals with closed shunts, and increased in the animals with unilateral ligated pulmonary arteries and with pulmonary hyperperfusion and hypertension. This suggests that increased levels of endothelin reflect abnormal haemodynamics rather than abnormal pathology. The high levels found in our newborns are in accordance with human studies,[23] and might implicate a neonatal predisposition of increased pulmonary vascular reactivity.

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In the present study, the circulating levels of von Willebrand factor were normal in both groups of animals undergoing surgical intervention. Clinical and experimental reports suggest that increased levels of this factor reflect endothelial damage or dysfunction.[24] In children and adults with established hypertensive pulmonary disease, increased levels have also been reported.[24,25] In this porcine model of moderate hypertensive pulmonary disease, the circulating level of von Willebrand factor was not found to be a marker of the disease.

In conclusion, by creating 3 mm central aorto-pulmonary shunts, or by unilateral pulmonary arterial ligation, we have created models of pulmonary vascular disease in newborn pigs subsequently surviving to three months of age. The shunts resulted in pulmonary hypertension and hyperperfusion, with subsequent normalisation due to spontaneous closure. Unilateral pulmonary arterial ligation resulted in sustained hyperperfusion and moderate hypertension in the lung fed by the patent pulmonary artery. Morphometrical analysis showed increased muscularity of the pulmonary arteries in both groups by three months of age, and the circulating levels of endothelin were increased in the group undergoing ligation. These findings could be of importance for future studies of the pathogenesis of hypertensive pulmonary disease associated with congenital cardiac malformations.

Limitations of study

In the pig, the pulmonary circulation adapts quickly to extrauterine life.[13] Also, the pig is born without antibodies, and hence must have colostrum for survival. Since the piglets could not go back to the sow after surgery, the piglets were left with the sow for their first 48 hours of life. At 48 hours, pulmonary arterial pressures and vascular resistance have already decreased significantly compared to the values immediately after birth. High levels of endothelin, nonetheless, were found in our newborns, implying that the pulmonary circulation was still in a state of newborn immaturity. In addition, significant changes of the pulmonary vasculature were subsequently achieved.

The Fick principle was used to calculate flows and vascular resistances, using an estimate for consumption of oxygen. Hence, changes within the specific inaccuracy of the method are not found, increasing the risk of β error. Obviously, haemodynamic changes beyond the inaccuracy of the Fick technique were aimed at in the established models. The Fick technique itself, therefore, was considered sufficient for evaluation of the induced haemodynamic changes.

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