Effect of sildenafil on right ventricular performance in an experimental large-animal model of postcapillary pulmonary hypertension

Effect of sildenafil on right ventricular function

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Abstract

Right ventricle (RV) dysfunction is a main determinant of morbidity and mortality in postcapillary pulmonary hypertension (PH). However, currently there are not available therapies. Since reduced nitric oxide (NO) availability and cyclic guanylate monophosphate (cGMP) levels are central in this disease, therapies targeting the NO pathway might have a beneficial effect on RV performance. In this regard, sildenafil has shown contradictory results. Our objective was to evaluate the effect of sildenafil on RV performance in an experimental pig model of postcapillary PH induced by a fixed banding of the venous pulmonary confluent. Animals were evaluated by right heart catheterization (RHC) and cardiac magnetic resonance (CMR) before randomization and after 8 weeks on sildenafil (n=8) or placebo (n=8), and myocardial tissues were analyzed with histology and molecular biology. At the end of the study, animals receiving sildenafil showed better RV performance as compared with those on placebo (improvement in RV ejection fraction of 7.3±5.8% vs -0.6±5.0%, p=0.021) associated with less apoptotic cells and gene expression related with reduced oxidative stress and increased anti-inflammatory activity in the myocardium. No differences were observed in pulmonary hemodynamics. In conclusion, in a translational large animal model of chronic postcapillary PH, sildenafil improved RV systolic function independently of afterload. Further research with pharmacological approaches able to manipulate the NO-cGMP axis are needed to confirm this potential cardioprotective effect.

Abbreviations

BSA body surface area

CMR cardiac magnetic resonance

cGMP cyclic guanylate monophosphate

ECV extracellular volume

HF heart failure

HFpEF heart failure with preserved left ventricle ejection fraction

HFrEF heart failure with reduced left ventricle ejection fraction

LHD left heart disease

LV left ventricle

MOLLI modified Look-Locker inversion-recovery

NO nitric oxide

PA pulmonary artery

PAH pulmonary arterial hypertension

PAP pulmonary artery pressure

PAWP pulmonary artery wedge pressure

PDE-5 phosphodiesterase type 5

PH pulmonary hypertension

PVR pulmonary vascular resistance

RHC right heart catheterization

ROIs regions of interest

RV right ventricle

Brief commentary

Background: Right ventricle (RV) dysfunction in postcapillary pulmonary hypertension (PH) is critical due to prognostic implications and the absence of available therapies. This study focuses on the potential effect of sildenafil on RV performance in an

experimental large-animal model of postcapillary PH evaluated by hemodynamics, magnetic resonance and techniques of histology and molecular biology.

Translational significance: In our translational large-animal model of chronic postcapillary PH, sildenafil prevented progressive RV systolic dysfunction independently of afterload and was associated with less apoptotic cells and changes in the profile of gene expression related to reduced oxidative stress and inflammation in the myocardium.

Introduction

Pulmonary hypertension (PH) secondary to left heart disease (LHD), currently classified as group 2 PH, is characterized by increased venous pulmonary pressure (postcapillary PH) and represents by far the most frequent etiology of PH[1]. It is estimated that approximately 50% of patients with chronic heart failure (HF), either with preserved (HFpEF) or reduced (HFrEF) left ventricular (LV) ejection fraction, have secondary PH[2]. Of them, 12-38% have combined pre and postcapillary PH[3, 4], a more severe pulmonary hemodynamic status that shares molecular, genetic and pathological characteristics with pulmonary arterial hypertension (PAH)[5]. In HF patients, the presence of PH results in more severe symptoms, worse exercise tolerance and poorer prognosis[6, 7].

Secondary right ventricle (RV) dysfunction is a main determinant of morbidity and mortality in HF, even stronger than the magnitude of pulmonary artery pressure (PAP) elevation[6]. Initially, there is a compensatory phase characterized by an increase in wall thickness and contractility, followed by RV dilatation, ventriculo-arterial uncoupling and finally RV dysfunction leading to clinically overt RV failure. Several pathophysiological mechanisms have been described in the “maladaptive” remodeling of the RV[8], that affect myocardial perfusion, metabolism and extracellular matrix

structure with increased inflammation, oxidative stress, apoptosis and fibrosis. However, the mechanisms involved in the failing RV and the potential for pharmacological intervention are only partially understood.

No single pharmacological approach has shown a consistent benefit in PH secondary to HF so far[1]. Since reduced nitric oxide (NO) availability and cyclic guanylate monophosphate (cGMP) levels are central in regulating endothelial function in PH secondary to HF[9], several studies have focused on targeting the NO pathway[10]. In this regard, Sildenafil, a phosphodiesterase-5 (PDE-5) inhibitor, has shown contradictory results regarding clinical outcomes and hemodynamic effects[11-15], that might be partially explained by the heterogeneity of the populations included and differences in the physiology of HFpEF and HFrEF. Although all these previous clinical studies mainly focused on hemodynamics and clinical outcomes, some reported an increase in tricuspid annular systolic excursion[13,14], an easily-obtained echocardiographic index of RV function. We hypothesized that a drug capable to increase NO availability and cGMP levels, and thereby increase protein kinase G, may have a direct beneficial effect on RV adaptation to pressure overload (by inhibiting pathways of pathological remodeling[16] and reducing stiffness[17, 18]), independent from its influence on the LV. Thus, our objective was to evaluate the effect of sildenafil on RV performance in an experimental model of postcapillary PH induced by a fixed banding of the venous pulmonary confluent, and therefore with normal LV function. Cardiac magnetic resonance (CMR), the gold-standard imaging technique for RV assessment, right heart catheterization (RHC), histology and molecular biology analysis were used.

Methods

All animal procedures were performed in castrated-male Large-White pigs. The study was approved by the Institutional Animal Research Committee and carried out in compliance with the Guide for the Care and Use of Laboratory Animals (Spanish RD 53/2013 and European Directive 2010/63/UE). Before any procedure, anesthesia was induced by intramuscular injection of ketamine (20mg/kg), xylazine (2mg/kg), and midazolam (0.5mg/kg). Buprenorphine (0.3mg/kg) was used for analgesia and animals were intubated.

Chronic PH was generated by surgical nonrestrictive banding of the confluent of both inferior pulmonary veins through a right minithoracotomy in 4 week-old pigs, as previously described in detail[19]. This approach made it very well tolerated, as it produces no initial hemodynamic disturbance, and generates progressive chronic PH as the animal grows that becomes stable from the second month onwards, associated with typical PH features on histopathology and CMR[19, 20], and has already been used to test potential therapies[21, 22]. Two months after surgery, animals were evaluated by RHC and immediate comprehensive CMR, and these measurements constituted the baseline hemodynamic and RV performance status. Subsequently, animals (all with confirmed PH) were randomized to receive sildenafil or placebo. Sildenafil was started on 25mg twice daily during 2 weeks and titrated to 50mg twice daily for the following 6 weeks. At the end of the protocol, animals were re-assessed by RHC and CMR, and underwent euthanasia by overdose of pentobarbital sodium to have their lungs and heart evaluated histologically. The histological study involved a morphometric study of the lung parenchymal vasculature and the evaluation of biventricular myocardial fibrosis, hypertrophy, vascularization and apoptosis. Isolation of mRNA and quantitative real-time PCR were performed for major pathways involved in vascular and cardiac remodeling in samples from the RV, LV and pulmonary artery (PA). Investigators performing and analyzing all RHC, CMR and histological studies

were blinded to treatment allocation; a veterinarian not involved in the study was responsible for the randomization and was the only person aware of group allocation.

Right heart catheterization

RHC was performed using a Swan-Ganz catheter (Braun, Kronberg, Germany) inserted through the femoral vein and positioned under fluoroscopy. Hemodynamic measurements included right atrial pressure, systolic, mean and diastolic pulmonary arterial pressure (PAP), pulmonary arterial wedge pressure (PAWP), and RV cardiac output (thermodilution method). Systemic blood pressures were monitored with a femoral arterial cannula (Arrow, Reading, PA, USA). Pulmonary vascular resistance (PVR) was calculated as the difference between mean PAP and LV end-diastolic pressure (quantified with a pigtail catheter instead of PAWP because of variation of PAWP amongst pulmonary lobes) divided by the cardiac output, in Wood units. Due to the significant increase in animal body weight between baseline and end-of-protocol measurements, cardiac output and PVR were indexed by body surface area (BSA) estimated by the Brody's formula[23].

Cardiac magnetic resonance

Acquisition

All studies were performed on a 3.0-T TX Achieva scanner (Philips Medical Systems, the Netherlands), equipped with a 32-channel cardiac phased-array surface coil and retrospective electrocardiographic gating during spontaneous ventilation. For cine imaging of the RV, a steady-state free precession sequence was used to acquire 10–15 contiguous short-axis slices covering both ventricles from base to apex, and reconstructed into 25 cardiac phases each for the evaluation of ventricular volumes and function. Two-dimensional flow imaging (phase-contrast) was performed perpendicular to the main PA with a velocity-encoded gradient echo sequence. Late gadolinium enhancement-CMR images were acquired 10 to 15 min after intravenous

administration of 0.15 mmol/Kg gadobutrol (Gadovist, Bayer Hispania S.L.) using a standard segmented inversion-recovery fast gradient-echo pulse sequence matching cine images. The inversion time was adjusted to null normal myocardium. The T1 mapping sequence (modified Look-Locker inversion-recovery [MOLLI]) was acquired before contrast administration and after equilibrium contrast. The acquisition protocol has been detailed elsewhere[20].

Analysis

All CMR analyses were performed using specialized software (Extended MR Workspace®; Philips Heathcare for animal studies) in a blinded fashion. On cine images, biventricular endocardial contours were manually traced in end-diastole and end-systole and Simpson's method was used to calculate volumes and ejection fractions. Biventricular epicardial contours in end-diastole were additionally traced to calculate biventricular masses. RV trabeculations were included within the blood pool and the interventricular septum was adjudicated to the LV mass. For phase-contrast analysis, the inner contour of the main PA was outlined in each cardiac phase to quantify the minimum and maximum areas, average velocities and stroke volumes. Ventricular volumes and masses and PA areas were adjusted to BSA. Regions of interest (ROIs) were drawn on T1 maps in the myocardial anterior and inferior RV insertion points, septum, LV lateral wall, RV free-wall and LV cavity blood pool before contrast administration and after equilibrium contrast[20]. Native T1 values were obtained from the pre-contrast T1 map, and extracellular volume (ECV) values were calculated as previously described[24]. T1 mapping images were acquired with high in-plane resolution to facilitate drawing the ROI inside the myocardium, and heart rate was updated before every MOLLI acquisition to define the correct trigger delay to minimize spatial misregistration.

Histology

All histological analyses were performed by two independent investigators blinded to allocation arm using ImageJ software. Pulmonary vascular remodeling was studied by double immunohistochemistry with anti-vonWillebrand factor to identify endothelial cells (1:200, A0082; Dako, Carpinteria, CA) and anti-smooth muscle actin to identify smooth muscle cells (1:100, M085101; Dako) antibodies, and Verhoeff-Van Gieson stain to identify elastic fibers. Morphometric analysis of ten randomly chosen arteries, five small arteries (<100 μm diameter) and five intermediate arteries (100-400 μm diameter) per histological section from the inferior and superior pulmonary lobes, were performed. The quantification of collagen content was performed using a picrosirius red stain in 5 myocardium samples from each ventricular region per animal (septum and LV and RV lateral walls). Cardiomyocyte area was analyzed by double immunofluorescence technique using wheat germ agglutinin (1:500, W834, Invitrogen, Eugene, OR) and cardiac troponin I (1:200, sc-15368, Santa Cruz Biotechnology, Santa Cruz, CA) as primary antibodies from 5 randomly chosen zones per histological section and 10 representative cardiomyocytes per zone from both ventricles. Vascularization density and apoptosis were semi-quantitatively analyzed in both ventricles, using isolectin B4 staining (1:25, B1205, Vector Laboratories Inc., CA, USA) and the In Situ Cell Death Detection Kit, Fluorescein (TUNEL technique) (Roche Applied Science, Indianapolis, IN), respectively (Supplemental Figure S1). Vascular density scoring (on an ordinal scale of normal, medium and low) was used under 630X magnification of the full histological section per animal. Apoptosis scoring (on an ordinal scale of minimal, mild and moderate) was used under 630X magnification of the full histological section per animal. A minimal score was defined as one or two TUNEL-positive cells per visual field; a mild score as more than two TUNEL-positive cells per visual field and moderate score as several areas with more than ten TUNEL-positive cells per visual field.

Molecular biology analysis

The effect of sildenafil on PDE5 and PDE6, and subsequent increase in cGMP in the LV and RV myocardium and PA were compared in both groups. Levels of cGMP were determined in tissues homogenates using commercially available enzyme-linked immune assay (ELISA, Cayman Chemical, Europe). Isolation of mRNA and quantitative real-time PCR (qPCR) were performed for major pathways involved in vascular and cardiac remodeling in samples from the RV, LV and PA. The amount of complementary DNA transcript copies in each tissue from sildenafil-treated pigs was established relative to the reference sample (controls) and expressed as Log2 of fold change. Total RNA was isolated from the PA and right and left ventricles using Trizol™ Reagent (Ambion, ThemoFisher). Total RNA concentration was determined by Qubit™ 3.0 Fluorimeter (ThermoFisher) and purity was assessed by Nanodrop™ One Spectrophotometer (ThermoFisher). Equal amounts of total RNA (2µg) from each tissue was reverse-transcribed to complementary DNA (cDNA) in the presence of MuLV reverse transcriptase, oligo(dT) and random hexamers with High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ThermoFisher). The amount of cDNA for major pathways involved in vascular and cardiac remodeling was quantified in each sample on an ABI Viia7 Real-Time PCR System using the Sybr™ Green-based methodology (Applied Biosystems, ThermoFisher). Those pathways included important components in the regulation of extracellular matrix organization (MMP9, MMP2, TIMP2, TIMP3); apoptosis (CASP3, CASP9, BAX); neovascularization (VEGF, VEGFR2, NOS3); inflammation (CSF2, IL-10, IL-1β, TNFα, IFN, SDF-1, TLR, MyD88); fibrosis (IL-6, TGFβ, SMAD); hypertrophy (NFAT2, CalcA, ANP, BNP); and oxidative stress (NOX, p47-phox, SOD, GPx). Primers for each gene were designed based on porcine mRNA sequence (Supplemental Table S1). Measurements were performed using 50ng of cDNA in 10µl volume of SYBR® Select Master Mix (Applied Biosystems, ThermoFisher), with the following cycling parameters: 95C for 10 min, followed by 40 cycles of 95C for 15 s and 60C for 1 min. Cycle threshold (Ct) values obtained for each

gene were referenced to the average Ct (ΔCt) of ACTB and GAPDH genes and converted to the linear form using the term $2^{-\Delta Ct}$.

Statistical analyses

The distribution of continuous variables (shape, presence of outliers) was analyzed with graphical methods. For normally distributed variables, results are expressed as mean±SD; otherwise expressed as median (interquartile range). Categorial variables are expressed as absolute frequency(%). Comparisons between groups (baseline variables and changes) were performed by parametric methods (nonpaired Student t-test) or nonparametric methods (Mann-Whitney U test) as appropriate. Chi-square or Fisher's exact test were used to compare categorical variables as required. All p-values are two-sided and statistical significance was set at $p<0.05$.

Results

A total of 17 animals were required to achieve a final cohort of 16 with complete follow-up (n=8 sildenafil and n=8 placebo). One animal (allocated to placebo group) died early after randomization. One animal included in the final cohort (allocated to placebo group) died just after the second CMR assessment, without time to perform the RHC.

There were no between-group differences in hemodynamic status and RV performance at baseline (Table 1). At 8-week follow-up, animals receiving sildenafil showed significantly better RV ejection fraction (improvement in RV ejection fraction of 7.3±5.8% vs -0.6±5.0%, p=0.021), RV-arterial coupling and RV end-systolic volume, although there were no significant differences in pulmonary hemodynamics as compared to placebo (Table 1, Figure 1). In addition, LV ejection fraction also increased in the sildenafil group. No differences were observed in CMR-derived myocardial ECV parameters (Table 2).

As expected, sildenafil treatment in pigs significantly increased cGMP in the myocardium of both ventricles and in the PA, in comparison to controls (Figure 2). On histological analysis, there were no differences between groups in vascular remodeling in the lung parenchyma (Table 3). On the myocardium, there were no differences in the amount of collagen content (in line with the CMR-derived myocardial ECV parameters), the degree of cardiomyocyte hypertrophy or the capillary density in both ventricles (Table 4). However, a small but statistically significant shift towards less apoptotic cells were found in the RV myocardium of animals receiving sildenafil as compared with placebo (Figure 3). In addition, in samples from pigs under sildenafil treatment, a significant increased expression of TIMP2 was observed in the PA, whereas a significantly reduced expression of pro-oxidant sources of superoxide anion was observed in the myocardium (NOX1 and p47-phox in the LV; and NOX1 and NOX2 in the RV). Moreover, sildenafil increased the expression of superoxide dismutase (SOD1 and SOD2) in the RV, responsible for the degradation of superoxide anion. Additionally, a down-regulated expression of IL-6 in the LV and an increased expression of TGFβ in the RV were also observed (Figure 4).

Discussion

In the present study we evaluated the effect of 2-month treatment with sildenafil on RV performance in a large-animal model of chronic postcapillary PH. Animals receiving sildenafil showed an improvement in RV ejection fraction associated with less apoptotic cells and changes in the profile of gene expression related to reduced oxidative stress and inflammation in the myocardium.

In PH secondary to LHD, reduced NO levels can contribute to vascular and myocardial cell damage by increasing oxidative stress, apoptosis and inflammation[8]. In addition, RV ischemia due to decreased NO-mediated vasodilation of small intramyocardic arteries has been associated with increased superoxide production[25]. By inhibiting

PDE-5, sildenafil reduces cGMP degradation, and thereby increases the action of NO and its potential protective cardiovascular effects. Although PDE-5 is minimally expressed in normal RV myocardium, it has been shown to be markedly upregulated in human hypertrophied RV myocardium[26], increasing contractility . Similar data have been shown for the LV[27, 28].

Given the prognostic value of RV dysfunction in PH and the absence of available therapies for PH secondary to LHD, RV adaptation to pressure overload deserves specific attention as a therapeutical target. Experimental studies evaluating an eventual direct cardioprotective effect on the RV by sildenafil have shown controversial results. Schafer et al.[29] found prevention of RV hypertrophy and fibrosis in the model of PH induced by monocrotaline (associated with improvement in pulmonary hemodynamics) but not in the model of PA banding in rats. Similar observations were made by Andsersen et al.[30] suggesting that sildenafil prevented RV myocardial remodeling in PH through an indirect action via RV unloading and not through direct actions on RV myocardium. However, subsequent evidence by Borgdorff et al.[18] showed a reduction in RV fibrosis and improvement of RV diastolic function in established RV dysfunction generated by PA banding in rats. In the same direction, more recently, Rai et al [31] reported that both sildenafil and riociguat prevented RV failure due to pressure overload in mice with PA banding, despite having no effects on RV hypertrophy. The PA banding model may seem a viable option to evaluate the effect of sildenafil on the RV independently to its effect in RV afterload; however, it carries limitations, as it is known that the hypertrophic response generated by a fixed “mechanical” pressure overload significantly differs from that generated by PH where increased myocardial apoptosis, oxidative damage, fibrosis and capillary rarefaction has been demonstrated[32]. In a model of postcapillary PH induced by transverse aortic constriction in mice, Pradhan et al.[33] reported a beneficial effect on the RV with

sildenafil. Similarly, using the same model, Imai et al[34] showed that sildenafil was able to prevent the expression of genes related with RV hypertrophy and inflammation.

To our knowledge, ours is the first study to evaluate the effect of sildenafil on the RV in a large-animal model of chronic postcapillary PH. The pig model has several advantages, such as its anatomical and physiological similarities to humans and the possibility of using RHC and CMR (the gold-standard methodologies in clinical practice), that may facilitate the inference of results to patients. Specifically CMR is the best technique for the quantification of RV volumes and ejection fraction, the latter being a powerful prognosticator in PH[35], and it is also considered useful to assess PA-RV uncoupling what precedes RV systolic dysfunction[36]. In our model we observed that sildenafil reduced progressive PA-RV uncoupling and systolic dysfunction. Unexpectedly, sildenafil administration was not associated with a reduction either in systemic blood pressure, PAP nor PVR. In a recent study using this experimental model, the acute administration of 10 mg of sildenafil did cause a reduction in total PVR[37], so we interpret that chronic vs. acute administration and differences in the time elapsed between administration and PVR measurement could account for these different results. In addition, we did not found differences in cardiac index either assessed by RHC or CMR. Differences in cardiac geometry with greater dilatation of both RV diastolic and systolic volumes (but more significant for the RV end-systolic volume) in the control group may explain the differences in RV ejection fraction without significant changes in cardiac index. Also this smaller disturbance of RV geometry may explain the better LV ejection fraction observed at the end of the study, as consequence of the ventricular interdependence.

Clinical evidence regarding the potential benefit of sildenafil on RV performance in PH due to LHD is even more scarce and controversial[11-15]. This is in part due to the high complexity of the disease, the great heterogeneity of the populations included in the trials in terms of HF etiology (HFpEF, HFrEF, valve heart disease), severity and

type of PH (isolated postcapillary PH and combined pre and postcapillary PH), and the poor characterization of the RV function. This highlights the need for accurate characterization and phenotyping of PH-LHD patients to conduct further research focused on specific patient phenotypes and probably shift our attention from PAP to RV function. In this line, Kramer et al.[14] recently reported that in a subset of patients with combined pre and postcapillary PH with HFpEF, sildenafil showed improved RV tricuspid annular plane systolic excursion and clinical parameters. Previously, Guazzi et al[13] had similarly showed an improvement in RV performance as assessed by echocardiography in patients with PH and HFpEF with confirmed systolic RV dysfunction. Our study provides further evidence in the same direction to these aforementioned studies in HFpEF. Other pharmacological approaches to manipulate the NO-cGMP axis, such as guanylate cyclase stimulators[38,39] and β3-adrenergic receptor agonists[21,40], have shown promising results.

Some limitations should be acknowledged. The experimental model used in the current study reproduces most pathology changes in the pulmonary circulation and the secondary RV adaptation of combined pre and postcapillary PH, as previously reported[19]. Although RV ejection fraction at baseline and at the end of the protocol (2 and 4 months after surgery, respectively) was only mildly reduced, our previous model characterization demonstrates that it is significantly lower as compared with healthy pigs[19]. However, leaving apart the known ventricular interdependence[41], the LV systolic and diastolic function remains normal and thus, it does not represent the entire spectrum of PH secondary to LHD. Animals were treated with 50 mg twice daily, a daily dose similar to that used in patients, during a period of 2 months. A longer period under treatment might have resulted in the detection of further changes at the level of molecular biology and histology, as previously shown by others[33,34]. In addition, sildenafil was initiated once PH was confirmed (animals being approximately 3-month old, and therefore considered adults), but the trigger for PH development started in the

pediatric phase, which might have an impact on RV adaptation. We used castrated-male pigs in order to avoid the potential effect of sex hormones in PH and RV adaptation[42], we acknowledge that both RV adaptation and the effect of drug therapies may differ between sexes. The sample size was small and, as we assessed just two groups (treated with sildenafil and control), we did not correct for multiple comparisons but we assume that multiple variable comparisons may increase the risk of type I error. In summary, the strength of our study is the use of state-of-the-art techniques as RHC and CMR in a translational large-animal model of post-capillary PH. As weakness, we acknowledge the small sample size, the relatively low impact of the experimental model on RV ejection fraction and the limited follow-up, which may partially explain the fact of not finding significant differences in RV hypertrophy and fibrosis (previously shown by others in small-animal models) and further changes at the level molecular biology. Therefore, our results should be considered as preliminary and further studies are necessary to confirm them.

In conclusion, in a translational large animal model of chronic postcapillary PH, sildenafil improved RV systolic function independently of afterload. Further research with pharmacological approaches able to manipulate the NO-cGMP axis are needed to confirm this potential cardioprotective effect.

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Figure 1. Changes in main hemodynamic and imaging parameters after sildenafil treatment in pigs. Bars represent mean and standard errors. P values compares pre-post changes between groups.

Figure 2. PDE5 activity in the porcine tissues. PDE5 activity and effectiveness of PDE inhibition by sildenafil were determined by the accumulation of PDE substrate cGMP. P values are expressed on top of each comparison.

Figure 3. Results of apoptosis in right and left ventricles.
On the left, representative histological section images of TUNEL technique (TUNEL-positive nuclei of apoptotic cells with magenta spot and nuclei counterstained with DAPI in blue) in the RV from control group (A), RV from sildenafil group (B), LV from control group (C) and LV from sildenafil group (D). On the right (E), bar chart showing a

significant decrease of apoptosis score in the RV of sildenafil group compared to control group.

Figure 4. Volcano Plot for differential gene expression.

Graphics display the differential expression of mRNA levels in PA, LV and RV of pigs treated with sildenafil in relation to control (untreated) pigs. Scattered points represent all genes studied plotted as the Log2 of fold change in the x-axis, whereas the y-axis is the probability that a gene has statistical significance in its differential expression, plotted as -Log10 of p value. Gene expression was considered significantly up- or down-regulated when they expressed at least 1.5-fold-change and $p<0.05$.

Table 1. Hemodynamic and imaging parameters at baseline and after 8-week treatment with sildenafil or placebo in pigs.

Variables	Baseline			At 8 weeks (end-of-protocol)		
	Control Group (N=8)	Sildenafil Group (N=8)	p	Control Group (N=8)	Sildenafil Group (N=8)	p*
Weight, Kg	32.5(26.0-34.5)	30.5(26.1-31.4)	0.442	49.8(46.9-53.6)	52.8(49.8-56.8)	0.065
Body surface area, m^2	0.88(0.76-0.91)	0.84(0.77-0.86)	0.442	1.15(1.11-1.21)	1.19(1.15-1.25)	0.105
Heart rate, bpm	78.0(60.5-89.3)	76.5(64.8-85.5)	0.721	69.0(63.0-84.0)	65.0(55.5-69.3)	0.721
Oxygen saturation, %	86.0(82.5-89.5)	86.5(80.5-91.8)	0.878	94.0(93.0-96.0)	92.0(90.3-94.8)	0.232

Systolic BP, mmHg	120.0(113.3-124.8)	113.0(100.3-121.8)	0.195	122.0(109.0-128.0)	113.0(108.5-119.8)	0.779
Systolic PAP, mmHg	42.5(35.8-48.3)	44.5(32.8-60.8)	0.721	42.0(34.0-46.0)	47.0(37.5-56.5)	0.779
Diastolic PAP, mmHg	24.0(17.8-32.0)	26.5(19.3-31.5)	0.798	23.0(23.0-29.0)	23.5(20.3-34.3)	0.613
Mean PAP, mmHg	31.0(28.3-37.3)	31.5(27.3-49.0)	0.798	33.0(26.0-34.0)	32.0(26.3-43.8)	0.694
Cardiac index, L/min/m^2	4.4(3.5-5.5)	4.4(4.0-4.9)	0.878	4.1(4.0-5.0)	3.8(3.5-4.3)	0.336
LV end-diastolic pressure, mmHg	8.0(5.8-9.8)	7.0(5.0-8.8)	0.442	8.0(7.0-10.0)	9.0(6.5-12.0)	0.463
Indexed PVR, WU/m^2	6.3(4.4-7.2)	6.8(4.8-9.2)	0.505	5.1(4.0-8.4)	6.6(3.2-9.4)	0.867
Ea/Emax	0.61(0.44-0.77)	0.72(0.60-1.07)	0.279	0.62(0.51-0.66)	0.55(0.30-0.70)	**0.009**
LV end-diastolic volume, mL/m^2	89.5(85.7-98.8)	91.1(85.8-102.9)	0.878	107.7(94.2-122.3)	85.7(76.1-109.4)	0.195
LV end-systolic volume, mL/m^2	36.1(30.7-38.9)	41.1(32.0-46.1)	0.382	45.5(38.8-48.9)	37.9(26.5-44.4)	0.065

LV ejection fraction, %	60.4(57.9-63.1)	56.8(52.6-61.6)	0.234	59.6(53.7-63.7)	61.9(56.4-65.2)	**0.038**
LV Mass, g/m^2	52.1(49.6-56.8)	50.4(48.3-62.0)	0.721	55.4(50.4-74.1)	60.7(51.9-64.3)	1.00
RV end-diastolic volume, mL/m^2	94.3(84.3-101.8)	94.9(88.8-106.7)	0.721	108.5(93.6-109.2)	90.3(85.4-100.3)	0.130
RV end-systolic volume, mL/m^2	39.6(33.7-49.6)	50.7(37.9-55.7)	0.279	46.0(39.0-54.0)	38.2(32.8-46.7)	**0.005**
RV ejection fraction, %	56.2(51.2-61.6)	52.8(44.2-57.9)	0.382	53.5(49.3-60.9)	59.5(53.5-60.7)	**0.021**
RV cardiac index by CMR, L/min/m^2	4.4 (3.4-5.2)	4.1 (3.6-4.3)	0.574	4.5 (3.9-5.2)	3.9 (3.6-4.2)	0.878
RV Mass, g/m^2	24.9(24.3-28.1)	25.6(22.8-30.1)	0.878	24.8(21.9-29.7)	25.1(21.3-29.5)	0.574

BP, blood pressure; PAP, pulmonary artery pressure; LV, left ventricular; RV, right ventricular; PVR, pulmonary vascular resistance. P*=p value compares pre-post changes between groups.

Table 2. Cardiac magentic resonance-derived myocardial native T1 and extracellular volumen values at baseline and after 8-week treatment with sildenafil or placebo in pigs.

Variables	Baseline	

	Control Group (N=8)	Sildenafil Group (N=8)	P	Control Gr
Anterior insertion point T1, ms	1092.5(1030.3-1183.0)	1087.5(1050.0-1138.5)	0.878	1042.0(100
Anterior insertion point ECV, %	32.4(23.7-40.5)	29.0(24.4-34.6)	0.645	30.3(24.
Posterior insertion point T1, ms	1083.0(996.0-1188.8)	1122.0(1101.3-1205.0)	0.234	1107.0(977
Posterior insertion point ECV, %	33.4(27.8-43.4)	38.1(29.7-40.3)	0.442	30.5(23.
Septum T1, ms	1097.5(1075.0-1131.8)	1111.0(1098.5-1126.8)	0.505	1074.5(102
Septum ECV, %	31.5(26.9-36.8)	30.5(27.3-33.7)	0.721	30.3(23.
LV lateral wall T1, ms	1123.0(1035.5-1180.8)	1068.0(1020.3-1089.8)	0.161	1033.5(102
LV lateral wall ECV, %	33.3(28.7-37.5)	32.5(24.2-36.1)	0.505	31.5(29.
RV lateral wall T1, ms	1135.5(1051.8-1254.5)	1092.5(1073.0-1183.8)	0.328	1157.0(106
RV lateral wall ECV, %	38.0(30.6-45.8)	29.8(24.9-37.9)	0.130	40.7(33.

ECV, extracellular volume; LV, left ventricle; RV, right ventricle.

p*= p value compares pre-post changes between groups.

Table 3. Morphometric study of the lung parenchyma vasculature

			Inferior pulmonary lobe				Superior pulmonary lobe		
	Small arteries		Intermediate arteries		Small arteries		Intermediate arteries		

	Control group (n=7)	Sildenafil group (n=7)	p	Control group (n=7)	Sildenafil group (n=7)	p	Control group (n=7)	Sildenafil group (n=7)	p	Control group (n=7)	Sildenafil group (n=7)	P
Total area (μm^2)	3058.0 (1637.5-3489.5)	3346.6 (2485.0-4217.9)	0.259	12401.9 (10205.7-15807.4)	13888.2 (9751.7-16289.9)	0.620	2850.3 (2134.6-2917.9)	2608.9 (2495.4-3006.3)	0.902	13390.8 (10861.5-18086.2)	13812.6 (6942.3-22492.5)	0.628

Table 4. Results from histological study in porcine myocardium.

	Right ventricle			Left ventricle		
	Control group (n=7)	Sildenafil group (n=7)	p	Control group (n=7)	Sildenafil group (n=7)	p
% of collagen	11.5(11.1-12.2)	9.9(8.1-13.9)	0.779	7.7(5.5-8.5)	8.6(6.5-10.7)	0.232
Cardiomyocytes area (μm^2)	150.0 (127.8-182.0)	164.2 (104.2-172.8)	0.645	157.1 (143.5-187.4)	176.2(118.0-188.4)	1.000
Vascular density Semi-Q analysis	Low (2); Medium (3); Normal(2)	Low (2); Medium(3); Normal (3)	0.935	Low (1); Medium (1); Normal	Low (3); Medium (3); Normal (2)	0.187

				(5)		
Apoptosis Semi-Q analysis	Minimal (2); Mild(4); Moderate(1)	Minimal(7); Mild (1); Moderate(0)	**0.045**	Minimal (3); Mild (3); Moderate (1)	Minimal (2); Mild(6); Moderate (0)	0.281