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The Assessment of the Quality of the Graft in an Animal Model for Lung Transplantation Using the Metabolomics ^{1}H High-Resolution Magic Angle Spinning NMR Spectroscopy

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Standards are needed to control the quality of the lungs from nonheart-beating donors as potential grafts. This was here assessed using the metabolomics ^{1}H high-resolution magic angle spinning NMR spectroscopy. Selective perfusion of the porcine bilung block was set up 30 min after cardiac arrest with cold Perfadex®. Lung alterations were analyzed at 3, 6, and 8 h of cold ischemia as compared to baseline and to nonperfused lung. Metabolomics analysis of lung biopsies allowed identification of 35 metabolites. Levels of the majority of the metabolites increased over time at 4°C without perfusion, indicating cellular degradation, whereas levels of glutathione decreased. When lung was perfused at 4°C, levels of the majority of the metabolites remained stable, including levels of glutathione. Levels of uracil by contrast showed a reverse profile, as its signal increased over time in the absence of perfusion while being totally absent in perfused samples. Our results showed glutathione and uracil as potential biomarkers for the quality of the lung. The metabolomics ^{1}H high-resolution magic angle spinning NMR spectroscopy can be efficiently applied for the assessment of the quality of the lung as an original technique characterized by a rapid assessment of intact biopsy samples without extraction and can be implemented in hospital environment. Magn Reson Med 68:1026–1038, 2012. © 2011 Wiley Periodicals, Inc.

Key words: lung transplantation; graft quality; HRMAS; NMR spectroscopy; metabolomics

INTRODUCTION

An important disparity between the number of lung donors and patients waiting for lung transplantation limits the number of lung transplantations performed each year. One proposition to increase the donors' pool would be to extend the current donor criteria, in particular by allowing lung transplantation from nonheart-beating donors (NHBDs).

Lung preservation is challenging in the early hours of cardiac arrest. The first studies show that the lung tissue remains viable with the oxygen trapped in the alveoli, allowing direct oxygen diffusion to the parenchyma, regardless of blood flow, up to 2 h after cardiac arrest (1). In addition, experimental evidence shows that the lung functionality can be preserved for 3–6 h in situ by topical cooling followed by an ex vivo perfusion in the laboratory with a lung perfusion system (2). The main disadvantage of this method is related to the complexity of its clinical application. Indeed, the topical cooling does not allow the maintenance of the oxygen in the alveoli, because this technique causes collapse of the lung.

Therefore, we propose in our work to investigate the possibility of implementing a selective in situ perfusion of the small pulmonary circulation by a preservation solution while maintaining pulmonary ventilation. Perfadex® solution is widely used in the current practice, as it is reported to contribute to reduce primary graft dysfunction after lung transplantation (3–5). The quality of the graft was assessed using the metabolomics ^{1}H high-resolution magic angle spinning (HRMAS) NMR spectroscopy of the lung biopsy samples. This was assessed in parallel to biochemical and enzymatic measurements in bronchoalveolar lavage fluid (BALF) that helps to identify lung inflammation or injury including total protein, albumin, lactate dehydrogenase (LDH), alkaline phosphatase (AP), and myeloperoxidase (MPO) and functional measurements including the end tidal CO_2 ($etCO_2$).

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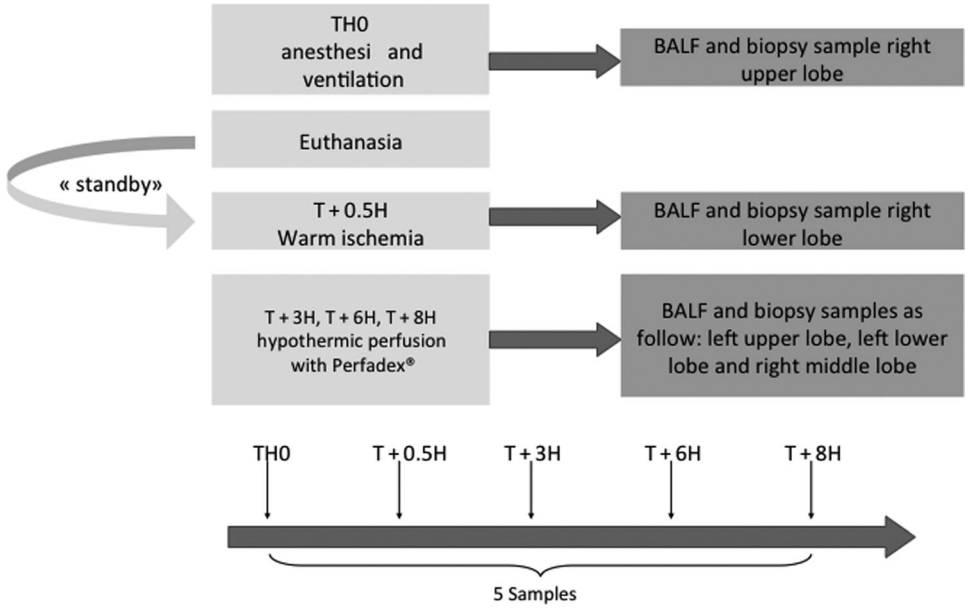

FIG. 1. Timeline of ischemia, different perfusion stages, and sampling throughout the experiment on six pigs used as models of NHBDs for lung transplantation.

METHODS

Animal Handling

Eight outdoor free-ranging domestic pigs with a mean weight of 27 ± 4 kg were used, two pigs for a preliminary study comparing the metabolic profile in the different lobes of the pig lung and six pigs for the assessment of the quality of the graft in NHBDs model for lung transplantation. These were housed in the animal facility at IRCAD (Institut de Recherche contre les Cancers de l'Appareil Digestif, Hôpitaux Universitaires de Strasbourg, France) in accordance with the French legislation rules of Ethics and the authorization of CREMEAS (The regional committee for ethics in experimentation using animals).

Premedication was performed with intramuscular ketamine (50 mg/kg) and azaperone (2 mg/kg), followed by intravenous induction of general anesthesia with propofol (3 mg/kg). Tracheal intubation was facilitated with pancuronium (0.2 mg/kg). Anesthesia was maintained with isoflurane and nitrous oxide (Abbott, Rungis, France) in 50% oxygen. Minute ventilation was initially adjusted to keep the end-tidal carbon dioxide concentrations between 35 and 45 mmHg using volume controlled ventilation and maintained constant after the aortic cross clamping. Monitoring consisted of electrocardiography, pulse oxymetry, capnography, invasive blood pressure measurement, body temperature with a rectal and a mediastinal temperature probe (Odam physiogard SM 785™, Sogemed SARL, Villefranche-sur-Saône, France), and pulmonary compliance and resistance. All data were continuously recorded (Aysis Carestation®, General Electric Healthcare©, Easton Turnpike Fairfield).

Surgical Procedure and Tissue Collection

Baseline blood and lung samples were taken in beating heart animals before inducing cardiac arrest. These included a heparinized sample for blood gas analysis and biopsies at T0 (fresh tissue) which were then treated to evaluate the effect of warm ischemia (Fig. 1).

Fraction of inspired oxygen was maintained after the stand-by period of 30 min following the induction of cardiac arrest, followed by maintaining the fraction of inspired oxygen at 50% all along the experiment. A right cervicotomy was combined to a median sternotomy to isolate the right jugular vein and to tape major intrathoracic vessels, i.e., both venae cavae and ascending aorta. This first step had the purpose of evaluating the feasibility of the in situ cold perfusion of the lung directly by both ventricles with open chest. The next step is to perfuse the lung percutaneously through the right jugular vein and the left carotid artery without sternotomy.

A Swan Ganz catheter (Swan-Ganz Combo V – Edwards Lifesciences LLC, Irvine, CA) was introduced through the right jugular vein and positioned into the main stem of the pulmonary artery under direct control to take a blood sample for blood gas analysis and to measure pulmonary arterial pressure.

Heparinization was allowed with 25,000 UI of standard heparin (Sanofi-Aventis, Paris, France). Then, a canulation was set up by inserting an arterial line into the right ventricle and a venous vent into the left ventricle (DLP 66120 20 Fr and DLP 66130 30 Fr, Medtronic, Minneapolis, MN). Cardiac arrest was induced by partial exsanguination, voiding 1 L of blood through the venous line. Cross clamping of both venae cavae and ascending aorta was performed with ligation.

The sampled blood was heparinized with 10,000 UI heparin (Sanofi-Aventis, Paris, France) and centrifuged at 2400*g* to obtain a packed red cell preparation without leukocytes of 300 mL. Steen solution™ was then added to the packed red cells with titration to a hematocrite of 15 ± 3%. Further additives were as follows: Imipenem 0.5 g (Tienam, Whitehouse, NS), insulin 20 IU (Actrapid, Lilly, Suresnes, France), and heparin 10,000 IU. The pH in the mixed solution was adjusted to a physiologic level with isotonic trometamol (trihydroxymethyl-aminomethane).

Following cardiac arrest, ventilation was stopped for a stand-by period of 30 min. At the end of the stand-by period, ventilation was resumed with identical parameters. Bronchoalveolar lavage (BAL) was performed in the posterior-caudal segment, and a biopsy was taken from the anterior-caudal segment (T + 0.5H). Pulmonary perfusion was initiated with isotonic saline using an ECMO pump (Sorin Group, Mirandola, Italy) with oxygenator (Custom Pack Strasbourg). Perfusion was continued with Perfadex (Vitrolife, Kungsbacka, Sweden) at 4–8°C during 3 h. Output of the pump was set at 0.5–0.7 L/min. The temperature of the perfusate was maintained with a refrigeration device (SARNS TCM II, 3M Health Care, Ann Arbor, MI) between 4 and 8°C.

After 3 h of perfusion with Perfadex, perfusion was switched to a second line containing autologous red blood cells suspended in Steen solution™ (Vitrolife). The latter was heated at 37°C. The oxygenator was then reversed to deoxygenate the Steen solution™ oxygenated by the lungs, by connecting a gas mix composed of CO_2 7%–O_2 7%–N_2 86% (Linde Group, Munich, Germany). Blood gas samples were taken on both sides of the membrane oxygenator.

Fiberoptic bronchoscopy with BAL was then performed in the left posterior-cranial segment, and a biopsy was taken from the anterior-cranial segment (T + 3H). All monitoring parameters of ventilation and pulmonary arterial pressure were recorded in real time.

Following these samplings and measurements, perfusion was switched back to the Perfadex® line, and a second 3-h period of perfusion with Perfadex at 4–8°C was initiated. At 6 h, we switched back to the Steen solution™ line, and a perfusion at 37°C was resumed. A similar sequence of samplings was made with BAL in the left posterior-caudal segment and a biopsy taken from the left anterior caudal segment (T + 6H), according to the rotation described above.

Following these samplings, we switched back to the Perfadex® line for a final period of hypothermic perfusion at 4–8°C during 2 h. The final samplings (BAL, biopsy) were taken from the right middle lobe at 8 h (T + 8H), after normothermic reperfusion with Steen solution™.

The lung biopsies and BAL collected at each time T + 3H, T + 6H, and T + 8H were performed rapidly, reducing the ischemic delay at maximum after resection. Biopsies were immediately frozen on dry ice and kept at −80°C until measurement. The BAL fluid was centrifuged at 1440*g* for 10 min at 4°C, aliquoted for further measurements of total protein, albumin, AP, and LDH, and stored at −80°C.

In total, five lung biopsies and BAL fluids were collected per pig: at T0 (fresh tissue), T + 0.5H (warm ischemia), T + 3H, T + 6H, and T + 8H (after perfusion; cold ischemia) (Fig. 1).

Samples Preparation for NMR Measurement

Each lung biopsy sample was prepared at −20°C as previously described (6) by introducing a biopsy of 15–20 mg into a disposable 30 μL KelF insert. To provide a lock frequency for the NMR spectrometer, 10 μL of D_2O was added to the insert. The insert was then sealed tightly with a conical plug and stored at −80°C until the HRMAS analysis. The insert insures that the entire biopsy sample is detected by the radiofrequency coil of the probe and that no leaks occur during the HRMAS analysis. Shortly before the HRMAS analysis, the insert was placed into a standard 4-mm ZrO_2 rotor and closed with a cap. The ensemble was then inserted into a HRMAS probe precooled at 4°C. All HRMAS experiments were performed at 4°C and were started immediately, after the temperature inside the probe had reached the equilibrium condition.

NMR Measurement

HRMAS spectra were recorded on a Bruker Advance III 500 spectrometer operating at a proton frequency of 500.13 MHz. The spectrometer is equipped with a 4 mm double resonance (^1H, ^{13}C) gradient HRMAS probe. A Bruker Cooling Unit regulates the temperature at 4°C. All NMR experiments were conducted on samples spinning at 3502 Hz.

For each biopsy sample, a 1D ^1H spectrum using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was acquired as previously reported (6,7). The interpulse delay between the 180° pulses of the CPMG pulse train was synchronized with the sample rotation and set to 285 μs ($1/\omega = 1/3502 = 285$ μs) to prevent signal losses due to B_1 inhomogeneities (8). The number of loops was set at 328 giving the CPMG pulse train a total length of 93 ms. Parameters for the CPMG experiment were sweep width 14.2 ppm, number of points 32k, relaxation delay 2 s, and acquisition time 2.3 s. A total of 128 free induction decays were acquired resulting in an acquisition time of 10 min. The free induction decay was multiplied by an exponential weighing function corresponding to a line broadening of 0.3 Hz prior to Fourier transformation.

All spectra were processed using automatic baseline correction routines. Spectra were referenced by setting the lactate doublet chemical shift to 1.33 ppm.

To help the assignment of metabolites, two-dimensional (2D) heteronuclear spectra were acquired. Typically, ^1H–^{13}C phase sensitive detection (echo/antiecho) heteronuclear single quantum correlations were performed using a 73 ms acquisition time with Globally Optimized Alternating Phase Rectangular Pulse (GARP) ^{13}C decoupling and a 1.5 s relaxation delay. A total of 116 transients were averaged for each of the 256 t1 increments (for a total acquisition time of 15 h; Refs. 6 and 7).

For NMR measurement, three experiments were performed on lung biopsy samples:

1. The effect of warm ischemia by comparing the metabolic profile between samples at T0 and T + 0.5H;
2. The metabolic evolution over time of control samples (T + 0.5 warm ischemia) under simulated ischemia at temperatures of 4 and 24°C. Between each NMR acquisition, the lung biopsy sample was placed out of the spectrometer in melting ice either at 4°C or at room temperature, i.e., 24°C for 3, 6, and 8 h of with the purpose of reproducing the situation of a conventional conservation at 4°C without perfusion, or mimicking the case of the organ stay in a potential donor after death at warm temperature chosen here at 24°C;

Table 1
Resonances Assignment in the 500 MHz ^{1}H HRMAS NMR Spectra of Pig Lung Biopsies

	Compound	Assignment	^{1}H chemical shift (ppm)	^{13}C chemical shift (ppm)	Concentration at T0 (fresh tissue, nmol/mg)	Concentration at T + 0.5H (warm ischemia, nmol/mg)
1	Acetate	Beta CH3	1.92	25.97	0.094	0.085
2	Alanine	Beta CH3	1.48	18.81	0.542	0.591
		Alpha CH	3.78	53.22		
3	Arginine	Alpha CH2	1.7	26.75		
		Beta CH2	1.92	30.15		
		Delta CH2	3.21	43.27		
4	Ascorbate	CH2(OH)	4.02	72.12	0.438	0.429
		C4H	4.52	81.25		
5	Asparagine	CH	4	54.15	4.920	5.113
		CH2	2.94	37.43		
		CH2	2.84	37.35		
6	Aspartate	CH2	2.7	39.17	0.870	0.779
		CH2 (d)	2.8	39.17		
		CH2 (d)	3.9	54.93		
7	Choline	N—(CH3)	3.21 (s)	56.75	0.316	0.316
		CH2	3.52	69.96		
		CH2	4.06	58.27		
8	Creatine	CH3	3.03 (s)	39.64	0.385	0.322
		CH2	3.93 (s)	56.36		
9	Ethanolamine	CH2	3.8	60.57		
		CH2	3.13	44.18	0.263	0.211
10	Fatty acids (a)	CH2	1.29	34.53		
		CH2	1.31	25.36		
	Fatty acids (b)	CH2	2.03	27.35		
		CH2	2.8	28.16		
		CH	5.33	130.51		
		CH	5.33	132.2		
	Fatty acids (a) and (b)	(*n*) CH2	1.29	32.4		
	Fatty acids (c)	CH2	1.6	27.3		
11	Alpha glucose	C4H	3.43	72.58		
		C1H	5.23	94.44		
		CH	3.84	59.12		
	Beta glucose	C3H,C5H	3.47	78.44	1.630	0.831
		C6H	3.75	63.44		
		C6H	3.89	63.44		
		C1H	4.65	98.7		
12	Glutamate	CH2	2.05	29.74	3.461	3.165
		CH2	2.34	35.95		
		CH	3.76	57.2		
13	Glutamine	CH2	2.14	29.52	0.460	0.516
		CH2	2.44	33.48		
		CH2	3.77	57.38		
14	Glutathione	CH2—CONH	2.54	33.79	0.277	0.286
		CH—NH2	3.78	46.03		
		CH—NH	4.57	58.55		
		CH2—SH	2.95	28.2		
15	Glycerol	1,3CH2OH	3.56	65.09		
		1,3CH2OH	3.65	65.09		
		CHOH	3.78	74.85		
16	Glycine	CH2	3.56 (s)	44.09	5.541	6.519
17	Glycogen	CH	5.40	102.55	0.295	0.277
		CH2	3.85	63.17		
		CH2	3.79	63.17		
		CH	3.43	72.08		
		CH	3.60	74.45		
18	Isoleucine	CH3	0.94	13.79		
		CH3	1.01	17.29		
		CH2	1.51	27.30		
		CH	3.65	62.34		

Table 1 (Continued)

	Compound	Assignment	^{1}H chemical shift (ppm)	^{13}C chemical shift (ppm)	Concentration at T0 (fresh tissue, nmol/mg)	Concentration at T + 0.5H (warm ischemia, nmol/mg)
19	Lactate	CH3	1.33	22.7	1.481	1.893
		CH	4.12	71.11		
20	Leucine	CH3	0.95	23.43		
		CH3	0.91	24.52		
		CH	1.70	26.78		
		CH2	1.70	42.37		
		CH	3.73	56.06		
21	Lysine	CH2	1.43	24.21	0.530	0.611
		CH2	1.71	29.10		
		CH2	1.89	32.56		
22	Methionine	CH3	2.12	16.61		
		CH2	2.18	32.72		
		CH2	2.64	31.50		
		CHNH2	3.85	56.84		
23	Myo-inositol	C5H	3.27	77.00	4.931	4.546
		C1H·C3H	3.54	73.84		
		C4H·C6H	3.61	75.06		
		C2H	4.06	74.85		
24	Nax	CH3	2.04	24.65		
25	Phenylalanine	CH2.6	7.30	131.81	0.036	0.067
		C4	7.37	131.49		
26	Phosphocholine	N(CH3)3	3.22	56.52	0.716	0.816
		CH2	3.60	68.98		
		CH2	4.16	60.60		
27	Phosphoethanolamine	CH2	3.98	63.05		
		CHNH2	3.22	43.35		
28	Proline	CH2	3.32	48.83		
		CH2	3.41	48.83		
		CH	4.10	64.39		
29	Syllo-inositol	All·HS	3.35	76.32	1.399	1.421
30	Serine	CH	3.84	59.12		
		CH	3.97	62.88		
31	Taurine	CH2—NH3	3.26	50.13	4.232	3.595
		CH2—SO3	3.42	38.06		
32	Threonine	CH	3.60	63.04		
		CH	4.24	68.67		
		CH3	1.32	22.30		
33	Tyrosine	CH	3.92	58.72	0.019	0.035
		CH3.5	6.87	118.50		
		CH2.6	7.16	133.37		
34	Uracil	C5H	5.78	103.65	0.001	0.005
		C6H	7.53	146.66		
35	Valine	CH3	0.98	19.16	0.106	0.201
		CH3	1.04	20.65		
		CH	2.30	31.94		

3. The effect of in situ perfusion on lung metabolites after cardiac arrest (T + 3H, T + 6H, and T + 8H); metabolic profiles were compared with those left at 4°C.

Metabolic evolution over time within each sample used the above-described parameters according to the following sequence of acquisition: 1D acquisition for 20 min (a total of 236 spectra) and 2D acquisition for 15 h (a total of 27 spectra). Evolution of the metabolomics was evaluated over time

Metabolite Quantification

A total of 35 metabolites were identified from these spectra in the pig lung (Table 1 and Fig. 2). Quantification was assessed for 24 metabolites. Results were expressed as nmol/mg of tissue. Chemical shifts for regions of interest were adapted to those of the literature (6).

Biochemical Measurements in BALF

LDH Assay

LDH activity was measured using the Quantichrom™ Lactate Dehydrogenase Kit (DLDH-100, BioAssay Systems, CA). This colorimetric LDH assay is based on the reduction of the tetrazolium salt (MTT) in a nicotinamide adenine dinucleotide (NADH)-coupled enzymatic reaction. The purple color formed was read at 565 nm. Results were expressed as IU/L and were linear from 5 to 45 IU/L.

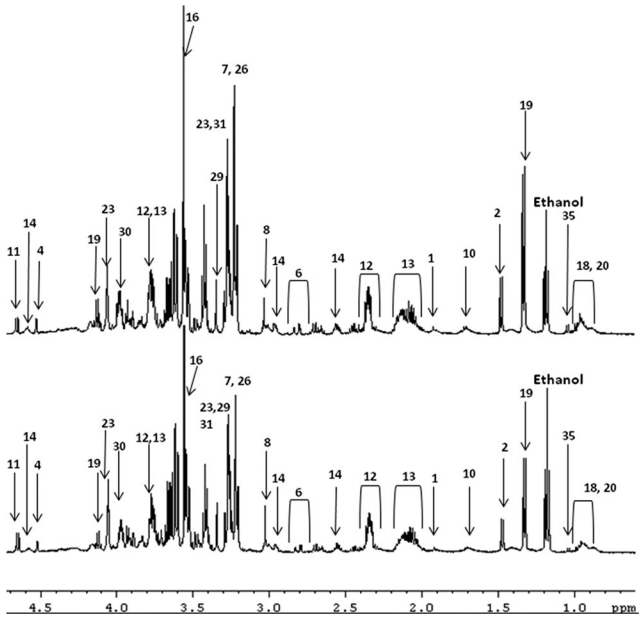

FIG. 2. 1D ^{1}H HRMAS CPMG spectra of lung biopsy samples collected at T0 (fresh tissue) and at T + 0.5H (warm ischemia). The numbers corresponding to the metabolite assignments are identified in Table 1. Ethanol is a pollution resulting from the surgical material. The metabolite constitution of either fresh tissue (T0) or tissue after warm ischemia (T + 0.5H) is similar.

AP Assay

AP concentration was determined using the Alkaline Phosphatase Colorimetric Assay Kit (Abcam®, Cambridge, UK) using *p*-nitrophenyl phosphate as a phosphatase substrate. The yellow color of the dephosphorylated substrate was read at 405 nm. Results were expressed as IU/mL and were linear from 1.5×10^{-3} to 7.5×10^{-3} IU/mL.

Total Protein Assay

Total protein concentration was determined using Bicinchoninic Acid Assay Kit (Sigma–Aldrich. Inc., St. Louis, MO). Concentrations were assayed according to bovine serum albumin standards obtained from Sigma Chemical Co. (St. Louis, MO). The results are expressed as g/L.

Albumin Assay

The albumin concentration was determined using the nephelometry method. An antihuman albumin antibody produced by immunization of rabbits with human highly purified albumin (Behring BNII, Siemens Kit) was used. The results are expressed as g/L.

MPO Assay

Lung biopsy samples were assessed for MPO activity using a standard kit (Calbiochem® #475911; EMD Chemicals, Inc., Merck Chemicals Ltd, Nottingham, UK). This colorimetric assay is based on the enzymatic cleavage of MPO by *o*-dianisidine. The yellow color obtained is read three times at 450 nm every 5 min. The last measurement was chosen for calculation. Results are expressed as IU/mg and were linear from 3×10^{-3} to 6×10^{-3} IU/mg of lung tissue.

Assessment of the Pulmonary Function Preservation

According to the physiological law:

$$VCO_2 = \text{interface surface/interface thickness} * \text{diffusion constant} * CO_2 \text{ gradient}$$

In this study, the precapillary carbon dioxide production was mimicked using the ECMO. The carboxylated blood substitute (Steen solution™) was then perfused into the pulmonary circulation to challenge the alveolocapillary membrane. $EtCO_2$ pressure was monitored using a GE Healthcare Aisys© carestation capnometer.

Statistical Analysis

An ANalysis Of VAriance (ANOVA) repeated measure followed by a Dunnett's multicomparison test was performed comparing the control samples (T + 0.5H) with the perfused samples and nonperfused samples both at 4 and at 24°C using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA; Table 2) confirming a significant difference between control samples (T + 0.5H) and nonperfused samples, whereas there is no significant difference between control samples (T + 0.5H) and perfused samples.

The spectral regions between 4.7 and 0.5 ppm of each 1D CMPG NMR, for experiments in the presence and absence of perfusion at 4°C, were involved as a principal component analysis (6) using subsequently the AMIX 3.8 software (Bruker GmBH, Germany) and SIMCA P (Umetrics AB, Umea, Sweden). The principal component analysis shows separation between two groups of samples without or with perfusion at 4°C. Results of biochemical analyses are given as means ± SEM.

RESULTS

Evaluating the Baseline Metabolic Profile (Six Pigs, $n = 8$)

A preliminary study on the different lobes of the pig lung was performed using NMR HRMAS confirming that there is no significant difference in the composition of metabolites between each region of the lung. These first analyses allowed identifying the 35 metabolites (Table 1) of the baseline metabolic profile of pig lung.

Evaluating Warm Ischemia (Six Pigs, $n = 12$)

Comparison of the spectral profiles performed on six pigs between lung samples collected at T0 and T + 0.5H (Fig. 2) shows an increase in metabolite levels (Table 1) for the 24 quantified metabolites. By contrast, the glucose levels showed a marked decrease between T0 and T + 0.5H. This is likely associated with the anaerobic conditions of the lung, related to the ventilation arrest. Indeed, in anaerobic glycolysis, cells use glucose as an energetic resource to form lactate, which, indeed, is increased in our conditions (Table 1). On the other hand, the glutathione (GSH) signal remained unaltered between T0 and T + 0.5H, and the uracil signal remained absent both at T0 and at T + 0.5H. This result indicates changes of metabolism in the lung during warm ischemia. These data led us to choose the concentration values from samples collected at T + 0.5H as our internal standards for further NMR measurements,

Table 2
Statistical Analysis on Metabolomics Data

	ANOVA Are means signif. different? ($P < 0.05$)	Dunnett's multiple comparison test: significant? ($P < 0.05$)								
		T + 0.5 vs. T + 3H (4°C)	T + 0.5 vs. T + 3H (24°C)	T + 0.5 vs. T + 3H (4°C) Perfused	T + 0.5 vs. T + 6H (4°C)	T + 0.5 vs. T + 6H (24°C)	T + 0.5 vs. T + 6H (4°C) Perfused	T + 0.5 vs. T + 8H (4°C)	T + 0.5 vs. T + 8H (24°C)	T + 0.5 vs. T + 8H (4°C) Perfused
Acetate	$P < 0.0001$	$P < 0.05$	$P < 0.05$	n.s.	$P < 0.05$	$P < 0.05$	n.s.	$P < 0.05$	$P < 0.05$	n.s.
Alanine	$P < 0.0001$	$P < 0.05$	$P < 0.05$	n.s.	$P < 0.05$	$P < 0.05$	n.s.	$P < 0.05$	$P < 0.05$	n.s.
Ascorbate	$P = 0.0021$	n.s.	n.s.	n.s.	n.s.	$P < 0.05$	n.s.	n.s.	$P < 0.05$	n.s.
Asparagine	$P = 0.0641$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Aspartate	$P < 0.0001$	$P < 0.05$	n.s.	n.s.	$P < 0.05$	$P < 0.05$	n.s.	n.s.	n.s.	n.s.
Choline	$P < 0.0001$	n.s.	$P < 0.05$	n.s.	n.s.	$P < 0.05$	n.s.	n.s.	n.s.	n.s.
Creatine	$P = 0.0005$	n.s.	$P < 0.05$	n.s.	n.s.	$P < 0.05$	n.s.	n.s.	$P < 0.05$	n.s.
Ethanolamine	$P < 0.0001$	n.s.	$P < 0.05$	n.s.	n.s.	$P < 0.05$	n.s.	n.s.	$P < 0.05$	n.s.
Glucose	$P < 0.0001$	$P < 0.05$	$P < 0.05$	n.s.	$P < 0.05$	$P < 0.05$	n.s.	$P < 0.05$	$P < 0.05$	n.s.
Glutamate	$P < 0.0001$	n.s.	$P < 0.05$	n.s.	n.s.	n.s.	n.s.	n.s.	$P < 0.05$	n.s.
Glutamine	$P = 0.0068$	n.s.	n.s.	n.s.	n.s.	$P < 0.05$	n.s.	n.s.	n.s.	n.s.
Glutathione	$P < 0.0001$	$P < 0.05$	$P < 0.05$	n.s.	$P < 0.05$	$P < 0.05$	n.s.	$P < 0.05$	$P < 0.05$	n.s.
Glycine	$P = 0.0002$	n.s.	$P < 0.05$	n.s.	$P < 0.05$	$P < 0.05$	n.s.	$P < 0.05$	$P < 0.05$	n.s.
Glycogen	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Lactate	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Lysine	$P < 0.0001$	n.s.	$P < 0.05$	n.s.	$P < 0.05$	$P < 0.05$	n.s.	$P < 0.05$	$P < 0.05$	n.s.
Myo-inositol	$P = 0.0100$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Phenylalanine	$P < 0.0001$	n.s.	$P < 0.05$	n.s.	n.s.	$P < 0.05$	n.s.	n.s.	$P < 0.05$	n.s.
Phosphocholine	$P = 0.0290$	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
Syllo-inositol	$P < 0.0001$	$P < 0.05$	n.s.	n.s.	$P < 0.05$	n.s.	n.s.	$P < 0.05$	n.s.	n.s.
Taurine	$P < 0.0001$	$P < 0.05$	n.s.	n.s.	$P < 0.05$	n.s.	n.s.	$P < 0.05$	n.s.	n.s.
Tyrosine	$P < 0.0001$	n.s.	$P < 0.05$	n.s.	n.s.	$P < 0.05$	n.s.	$P < 0.05$	$P < 0.05$	n.s.
Uracil	$P < 0.0001$	n.s.	$P < 0.05$	n.s.	$P < 0.05$	$P < 0.05$	n.s.	$P < 0.05$	$P < 0.05$	n.s.
Valine	$P < 0.0001$	n.s.	$P < 0.05$	n.s.	n.s.	$P < 0.05$	n.s.	n.s.	$P < 0.05$	n.s.

ANOVA reapeted meusure test followed by Dunnett's test compared control samples (T + 0.5H), perfused samples, and nonperfused samples at 4 and 24°C. The pairing is significant with $P < 0.05$.

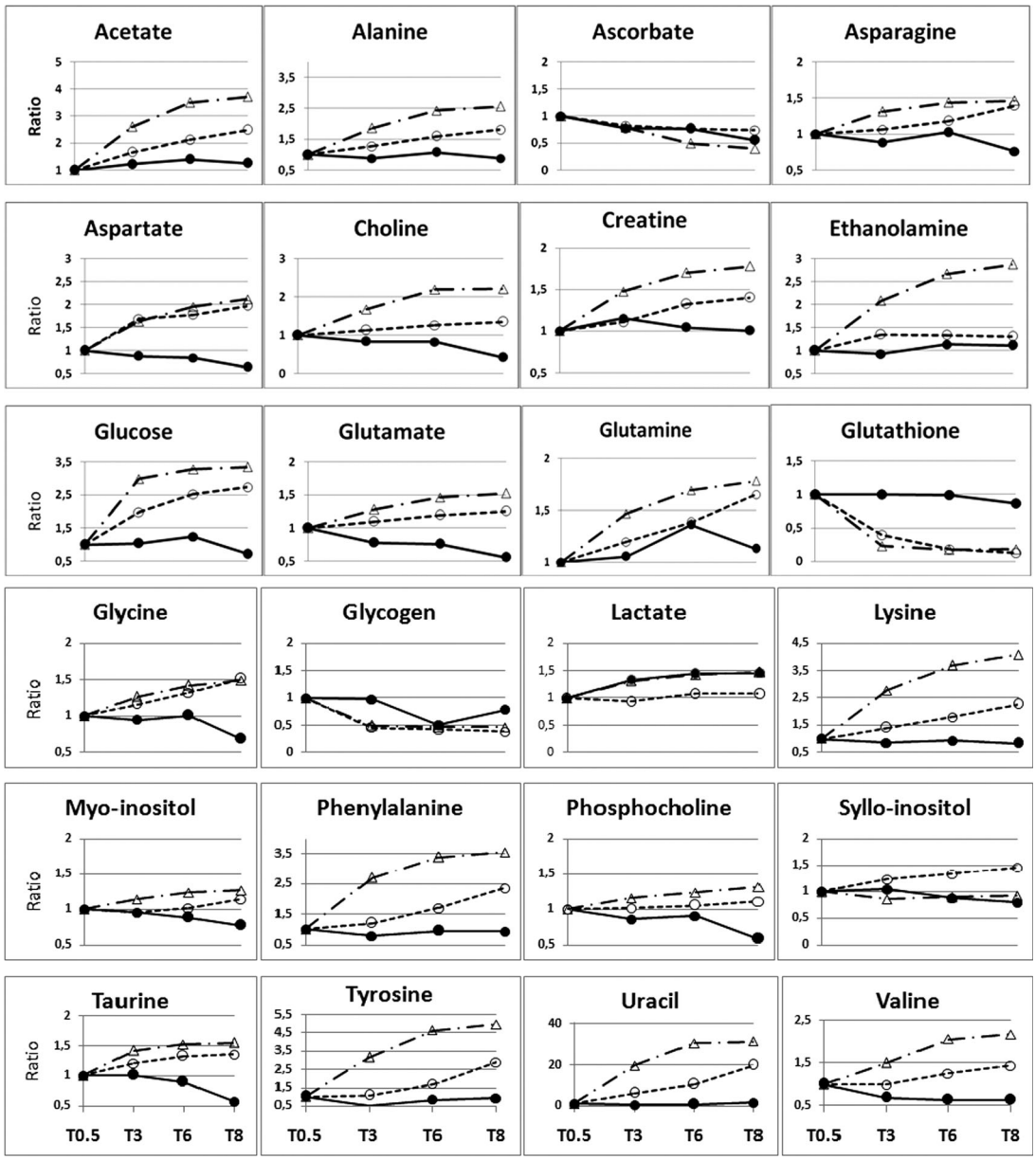

FIG. 3. Metabolites evolution over time in the absence of perfusion at 4 and 24°C and in the presence of perfusion at 4°C. Results are represented as a ratio of the control concentration (T + 0.5H). The symbols correspond to (●) perfusion at 4°C, (○) evolution in the absence of perfusion at 4°C, and (ρ) evolution in the absence of perfusion at 24°C.

because this corresponds to the time of warm ischemia reproducing a similar situation as occurs with a donor.

Evolution of ^{1}H NMR Spectroscopy Profiles over Time in the Absence of Perfusion (Six Pigs, $n = 12$)

Evolution of the metabolomics was evaluated over time under simulated ischemia at temperatures of 4 and 24°C. Between each NMR acquisition, the lung biopsy sample was placed out of the spectrometer in melting ice either at 4°C or at room temperature, i.e., 24°C for 3, 6, and 8h. The levels of the majority of the metabolites increased more importantly over time at 24°C than at 4°C (Fig. 3). The main changes were observed for glucose, lactate, alanine, choline, phosphocholine, and acetate. In addition, aspartate and myo-inositol increased more slowly than all others. Conversely, the ascorbate levels decreased over time more significantly at 24°C than at 4°C, and GSH totally disappeared after 3 h of ischemia (T + 3H) whether at 4 or 24°C (Fig. 3). These overall results reflect the necessity to preserve tissues at low temperature (with perfusion at 4°C), as cell damage is reduced. These results also give preference to results obtained with cold perfusion (4°C) as the standards to control our further experiments.

Evolution of ^{1}H NMR Spectroscopy Profile Under Perfusion (Six Pigs, $n = 18$)

NMR HRMAS measurements were then performed on samples collected after cold ischemia, i.e., T + 3H, T + 6H, and T + 8H of in situ perfusion with Perfadex®. Two metabolites reacted significantly at perfusion: GSH

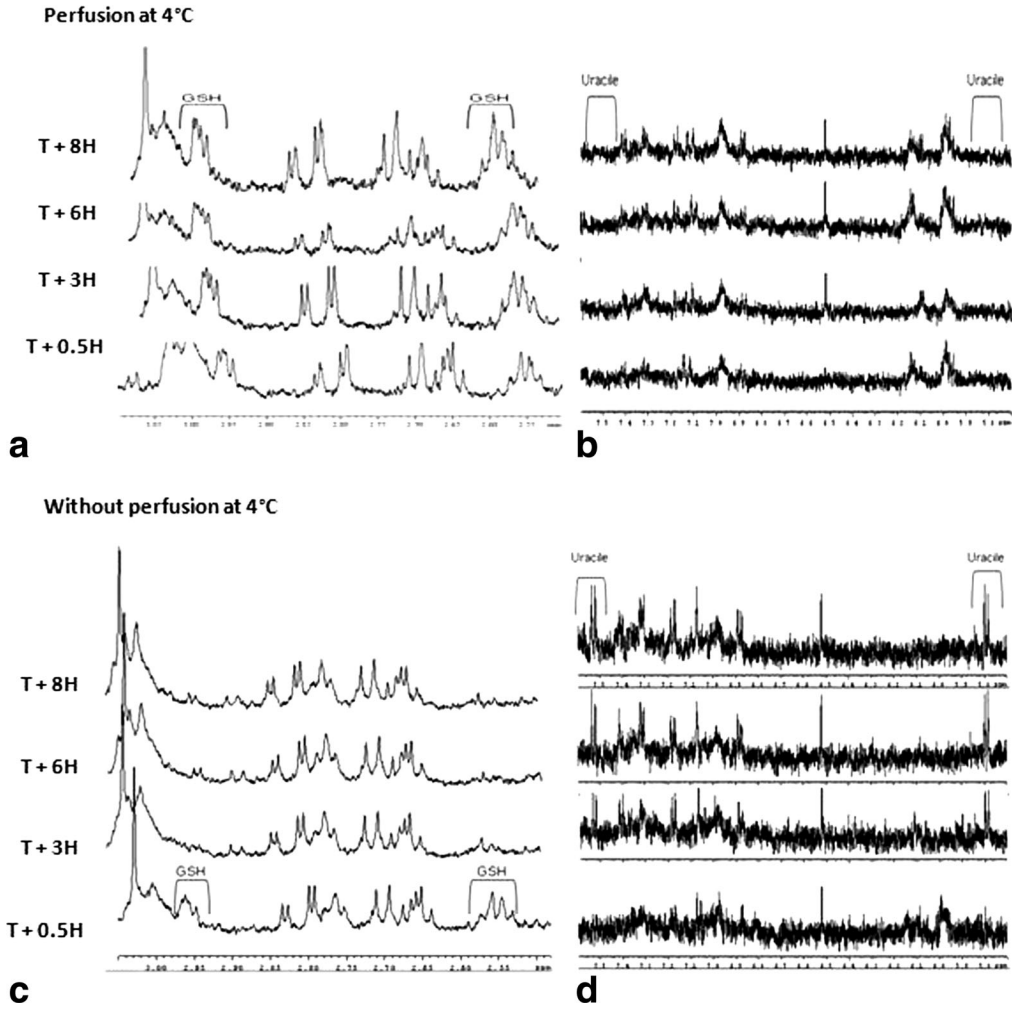

FIG. 4. 1D ^{1}H CPMG HRMAS spectra, at 4°C, of pig lung biopsy samples collected at T + 0.5H (warm ischemia). The experiments were performed at T + 0.5H, then at 3, 6, and 8H. Between each acquisition, the sample was taken out of the spectrometer and left at 4°C. The left spectrum corresponds to the region between 2.45 and 3.05 ppm, in which two signals of GSH (2.55 and 2.95 ppm) are observed. The GSH signals disappeared in the absence of perfusion over time at 4°C. The right spectrum corresponds to region between 5.70 and 7.80 ppm, in which two signals of uracil (5.78 and 7.78 ppm) are observed. The uracil signals appeared and increased over time at 4°C in the absence of perfusion. The GSH signals remained still stable over perfusion time. The right spectrum corresponds to region between 5.70 and 7.80 ppm, in which two signals of uracil (5.78 and 7.78 ppm) are detected. The uracil signals did not occur, when the lung was perfused.

and uracil. The GSH signal was stable throughout perfusion with Perfadex® (0.5–8 h; Fig. 4a), whereas it disappeared over time in the absence of perfusion (Fig. 4c). By contrast, uracil showed an opposite profile, as its signal was absent in the perfused samples all along the 8 h (Fig. 4b), whereas it increased over time at 4°C in the absence of perfusion (Fig. 4d).

In addition, principal component analysis corresponding to spectra obtained from samples perfused, nonperfused, and control samples (warm ischemia) shows separation between two groups of samples without or with perfusion at 4°C. This analysis also shows that samples after warm ischemia (control samples) are in the same region that the samples perfused at 4°C, demonstrating that there was no noticeable degradation at the starting point of our experiment (warm ischemia) and that the control samples after warm ischemia were not distinguishable from the perfused samples (data not shown).

Furthermore, comparison of NMR spectra in the presence or absence of perfusion at 4°C (Fig. 3) showed a significant increase in the concentrations of all other metabolites in samples without perfusion as compared with those undergoing the in situ perfusion. However, the lactate levels, as a metabolite of glucose, increased both in the presence and in the absence of perfusion. This may be linked to the ischemic delay between the collection of the biopsy and its freezing.

Biochemical Analyses (Six Pigs, $n = 30$)

MPO activity is an important parameter to assess neutrophil activation and therefore the presence of neutrophilic inflammation (9). MPO activity in lung biopsies was stable, with low values at T + 0.5H (3.7 ± 0.8 μUI/mg; Fig. 5a), remaining stable in samples collected after perfusion at T + 3H, T + 6H, and T + 8H: 3.0 ± 0.7, 3.2 ± 0.2,

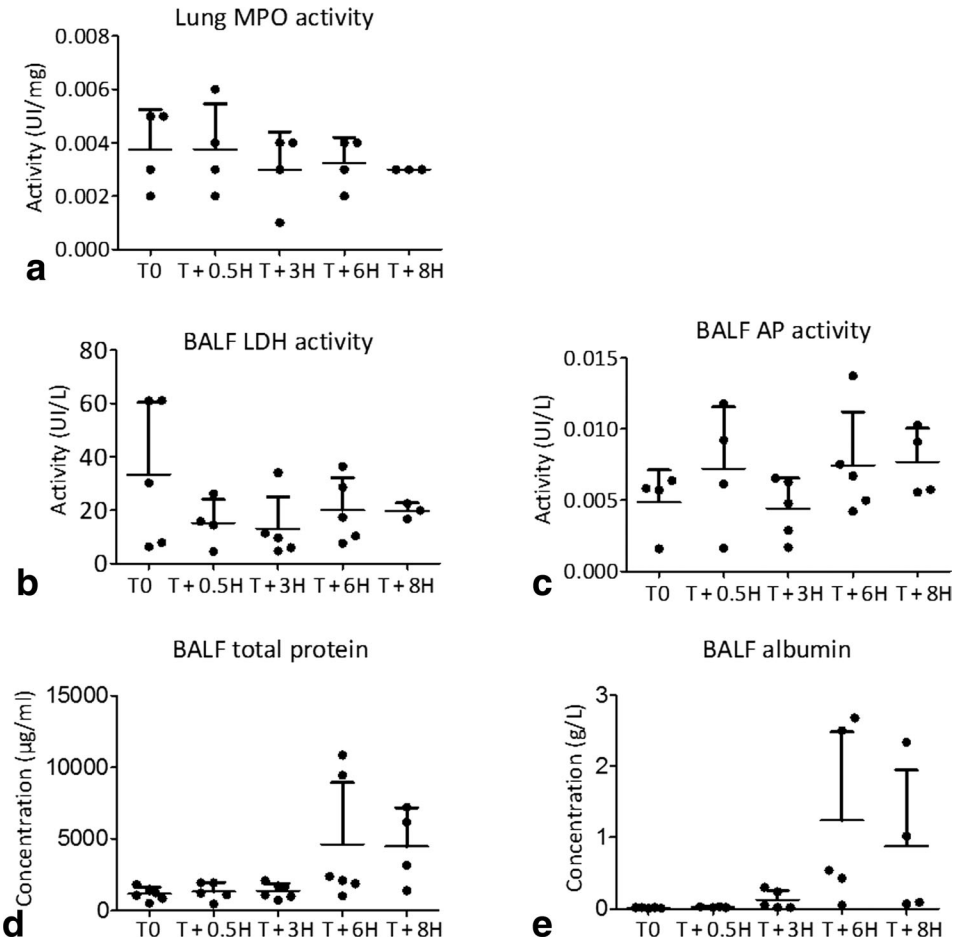

FIG. 5. Biochemical analyses: MPO activity measured on biopsy samples as a biomarker for neutrophil activation; BALF LDH activity measured as a biomarker of cell damage; BALF total protein and albumin concentrations measured as biomarkers of edema; and BALF AP activity measured as a biomarker for pneumocyte type II injury. Data are means ± SEM of 28 BALF and biopsy samples collected from $n = 6$ pigs.

and 3.0 ± 0.0 μUI/mg, respectively, indicating that no neutrophils were adherent to the pulmonary endothelium at euthanasia.

The LDH activity in BALF was measured as an indicator of nonspecific cellular damage (10). LDH activity from perfused lungs remained constant over time throughout the experiment (mean value: 20.3 ± 3.5 IU/ml; Fig. 5b).

The AP activity in BALF is largely used to investigate lung injury, as it is a specific indicator of type II pneumocytes damage (10). AP activity was stable over time within the perfused lung samples (mean value: 6.3 ± 0.0007 μUI/L; Fig. 5c).

Total protein concentration in BALF is an indicator of an increase of vascular leakage, leading to potential edema, or cell damage (11,12). Total protein concentration remained constant between T + 0.5H and T + 3H (Fig. 5d). At T + 6H and T + 8H, two outliers were noticed, as results of an increase in total proteins. This increase arose in two pigs, in which a pulmonary edema occurred during the surgical procedure. This result was confirmed, when albumin concentration was assessed (Fig. 5e). Albumin concentration remained stable in these two pigs with pulmonary edema between T + 0.5H and T + 3H, whereas it markedly increased after 6H and 8H of perfusion in parallel to the increase in total protein in BALF.

For all other animals, the levels of total protein and albumin remained constant as compared to control samples (T + 0.5H) throughout the experiment, indicating that no cell damage occurred over time within the perfused lung samples.

Functionality of the Perfused Lung (Six Pigs)

After induction of anesthesia, mean $etCO_2$ was 32 ± 3 mmHg. At sternotomy and Swan Ganz probe insertion, mean $etCO_2$ was 39.8 ± 1.0 and 34.5 ± 1.5 mmHg, respectively. After cardiac arrest and during each period of perfusion with Perfadex® (cold ischemia), mean $etCO_2$ was 0. At perfusion with red blood cells in Steen solution™, maximal $etCO_2$ was 10.1 ± 1.1, 9.3 ± 0.8, and 8.1 ± 1.8, respectively, during the first (T + 3H), second (T + 6H), and third (T + 8H) switch (Fig. 6). This $etCO_2$ description during the experimental in situ perfusion clearly indicates that a pulmonary transcapillary CO_2 diffusion still occurs, which suggests a preserved lung function for gas exchange during the 8H of cold perfusion.

These results are in accordance with the other functional measurements such as arterial pressure in oxygen (PaO_2), arterial pressure in carbon dioxide ($PaCO_2$), arterial oxygen saturation (SaO_2), pulmonary arterial pressure, and pulmonary arterial resistance (data not shown, they are the subject of another manuscript of our research team).

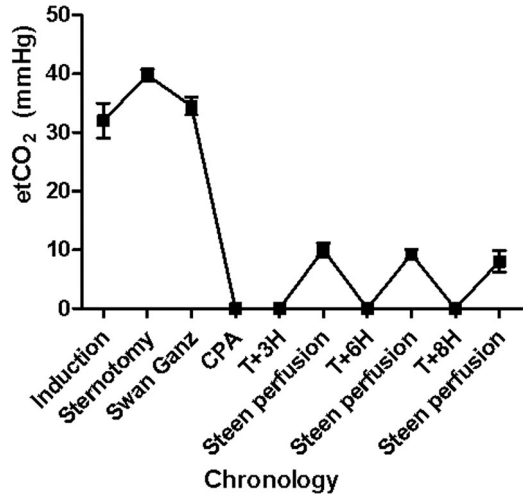

FIG. 6. Pulmonary functional preservation study of gas exchange in the lung model perfused with Perfadex®. The mean $etCO_2$ is expressed as mmHg. In the presence of erythrocytes in the Steen® solution perfused at 3, 6, and 8 h of cold ischemia, gas exchange was evidenced by the increase in $etCO_2$ pressure measured in the gas of the respiratory circuit used to ventilate the lungs. CPA: cardio pulmonary arrest.

DISCUSSION

We here report the metabolic profile of intact lung biopsy samples obtained from nonheart-beating pigs perfused with cold Perfadex® as measured by the metabolomics ^{1}H HRMAS NMR spectroscopy technique. This shows stability of the metabolic profile over perfusion time as compared to nonperfused lung samples. The quality of the lung was additionally ascertained (1) biochemically with more classical measures of lung damage and (2) functionally by the gas exchange measurements.

Several studies report the feasibility of lung transplantation from NHBDs. Egan et al. (13) first showed that dog lung remains viable for 2 h after cardiocirculatory arrest based on the measurements of the hemodynamic and gas exchange during the observation period of the survival of a canine allotransplantation. Transplantation from NHBDs was then reported by Steen et al. (2), followed by the first series of 17 transplantations of lungs from NHBDs by Gomez de Antonio et al. (14). These methods used organ preservation by ex vivo topical cooling during the first 2–4 h. We show here that a continuous hypothermic perfusion of the lung in situ enables preservation without morphological or functional deterioration as validated by our metabolomics, biochemical, and functional results for 8 h. Surgery was here performed with an open thorax to validate the method with direct canulation of the ventricles. These results open the field to a percutaneous method using puncture of the vessels of the neck. Such an evolution of the technique will reconcile between the necessity of cooling the lungs and the respect of the integrity of the donor. It may thus be proposed as a useful strategy for the donor lung preservation while waiting for authorization from legal administration and relatives.

The validation of the quality of the lung was here performed for the first time by HRMAS NMR spectroscopy. This is a well-established and powerful implement for obtaining metabolic information on biological intact tissues. It has been widely reported in studies to characterize malignant tissue (15,16). However, very few papers appeared in the transplantation field to study the quality of the graft (17,18).

The CPMG experiment here used consists in a succession of 180° pulses separated by an interpulse delay. In our experiments, the interpulse delay is short (285 μs) with respect to $1/J$ (J: homonuclear, H—H coupling constant). This value prevents the formation of a significant amount of antiphase magnetization. Therefore, under the conditions used for our experiments (total CPMG time of 285 * 328 = 93,480 μs = 93 ms), the intensity of all metabolite is preserved. Only a small effect due to T2 relaxation can be observed for the metabolites of interest. Furthermore, the CPMG experiment attenuate selectively the signals of compounds with short T2 (macromolecules and lipids).The metabolomics 1D ^{1}H NMR HRMAS used here allowed to identify 35 metabolites and to quantify 24 out of them, among which two had a significant behavior indicating they may be used as potential biomarkers of the quality of the lung: GSH and uracil.

We notice that the GSH signal was stable during perfusion with cold Perfadex®, whereas it disappeared over time in the absence of perfusion. GSH is an intracellular thiol present in all tissues, including the lung. It is well established that GSH is the most important endogenous antioxidant agent with functions including protection against oxidative damages (19,20). Low levels of GSH have been associated with high risk of lung damage and various diseases (21,22). The importance of the GSH system in the protective effect of the lung against cell damage has been demonstrated by studies showing enhancement of lung oxygen toxicity in animal models depleted of GSH (23). Our result of a stable GSH in our model of in situ perfused lung indicates that the protective antioxidant system is still active even after 8 h of cold perfusion and allows proposing use of our surgical perfusion technique before collecting the lung for transplantation.

On the other hand, we observe an opposite situation with the uracil signal. Indeed, the uracil signal was absent in the control samples and remained absent over time during cold ischemia with Perfadex® solution, whereas it markedly increased over time in the absence of perfusion whether at 24 or 4°C. Recent studies report a change in the levels of cellular purine or pyrimidine, such as uracil, following oxidative stress (24,25), indicative of cell damage. Cell stress generated by reactive oxygen species lead to the release of adenosine triphosphate and uridine triphosphate in the extracellular fluid. After 2 h, the adenosine triphosphate levels decrease resulting in its degradation to adenosine (24). Finally, the adenosine triphosphate production decreases under cell stress; this generates adenosine monophosphate (AMP) and adenosine diphosphate (ADP) which degraded into final metabolites. In the same way, for the uridine triphosphate which degrade to uracil. Although the experiment parameters of the 2D heteronuclear single quantum correlation did not allow visualizing the aromatic region of other nucleotide/nucleoside such as adenosine, guanosine, and tymidine, their presence could not be excluded.

Thereby, our result indicates that the perfusion has a positive effect on the quality of the lung, because cells have been preserved against oxidative disorders.

Thus, we may submit uracil as a stress marker with concentration levels increasing over time with the severity of cell damage, and GSH as a potential biomarker declining during stressed lung damage.

Additionally, an increased concentration of metabolites during oxidative stress is a well-established characteristic, as occurred in our study where glucose, lactate, choline, phosphocholine, alanine, and acetate concentrations increased. These metabolites are mainly involved in cell degradation and apoptosis, and are markers for cell damage, and our data showed that the metabolic profile of these metabolites was stable in the biopsy samples from lung perfused with cold Perfadex® up to 8 h of cardiac arrest, indicating that this technique of perfusion preserves the integrity of the lung cells.

Biochemical and enzymatic analyses complemented the NMR HRMAS metabolomic results. Stability of the MPO activity indicates that neutrophils are neither present nor activated within the lung biopsy samples after cardiac arrest. Analysis of cells from BALF confirmed that there was no infiltration of polymorphonuclear neutrophils (not shown). Furthermore, the stability of LDH and AP activities in the BALF also indicates that there was no cellular damage and type II cells conserved their integrity. For total protein concentration in BALF, except an increased concentration for two pigs with known edema, the concentration was stable, which was confirmed by albumin levels also increasing only in the two pigs with edema. This confirms that no cell damage occurred during the 8 h of perfusion in the nonheart-beating pigs.

Finally, the functional analysis of the $etCO_2$ indicates persistence of the pulmonary transcapillary CO_2 diffusion during the experimental cold Perfadex® perfusion. This CO_2 diffusion shows a persisting function in gas exchange. Therefore, this functional preservation of the alveolo-capillary gas exchange is consistent with a good preservation of the lung during in situ cold perfusion, which is in agreement with stable metabolomics and biochemical results for at least 8 h of cold perfusion with Perfadex®.

CONCLUSIONS

Thus, the metabolomics NMR HRMAS can be efficiently applied for the assessment of the quality of the lung as an original and a very reliable technique characterized by a rapid assessment (in 20 min), and may be easily implemented in any hospital environment. This first study in the porcine lung allowed the identification of GSH and uracil as potential biomarkers of the quality of the lung cells. These data with metabolomics as well as biochemical and functional results clearly show that the quality of the graft is maintained by in situ perfusion of the lung after cardiac arrest with a preservation solution at low temperature (4°C). As a next step, we will evaluate the quality of the lung graft after autologous transplantation to prove that this novel technique of in situ perfusion presented here in nonheart-beating pigs maintains the lung viable.

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