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ORIGINAL ARTICLE

Cold ischemia with selective anterograde *in situ* pulmonary perfusion preserves gas exchange and mitochondrial homeostasis and curbs inflammation in an experimental model of donation after cardiac death

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Summary

The aim of this study was to assess the functional preservation of the lung graft with anterograde lung perfusion in a model of donation after cardiac death. Thirty minutes after cardiac arrest, *in situ* anterograde selective pulmonary cold perfusion was started in six swine. The alveolo-capillary membrane was challenged at 3, 6, and 8 h with measurements of the mean pulmonary arterial pressure (mPAP), the pulmonary vascular resistance (PVR), the PaO_2/FiO_2 ratio, the transpulmonary oxygen output ($tpVO_2$), and the transpulmonary CO_2 clearance ($tpCO_2$). Mitochondrial homeostasis was investigated by measuring maximal oxidative capacity (V_{max}) and the coupling of phosphorylation to oxidation (ACR, acceptor control ratio) in lung biopsies. Inflammation and induction of primary immune response were assessed by measurement of tumor necrosis factor alpha (TNFα), interleukine-6 (IL-6) and receptor for advanced glycation endproducts (RAGE) in bronchoalveolar lavage fluid. Data were compared using repeated measures ANOVA. Pulmonary hemodynamics (mPAP: $P = 0.69$; PVR: $P = 0.46$), oxygenation (PaO_2/FiO_2: $P = 0.56$; $tpVO_2$: $P = 0.46$), CO_2 diffusion ($tpCO_2$: $P = 0.24$), mitochondrial homeostasis (V_{max}: $P = 0.42$; ACR: $P = 0.8$), and RAGE concentrations ($P = 0.24$) did not significantly change up to 8 h after cardiac arrest. TNFα and IL-6 were undetectable. Unaffected pulmonary hemodynamics, sustained oxygen and carbon dioxide diffusion, preserved mitochondrial homeostasis, and lack of inflammation suggest a long-lasting functional preservation of the graft with selective anterograde *in situ* pulmonary perfusion.

Introduction

Lung transplantation remains the ultimate therapeutic option for patients suffering end-stage pulmonary diseases [1,2]. In France, between 2007 and 2010, the number of patients on the waiting list for lung transplantation has increased from 131 to 178 (36%, Agence de la Biomédecine, 2010, available from: http://www.agence-biomedecine.fr/agence/nationaux.html) mainly as a result of the scarcity of suitable donors. This shortage of available lung grafts translates into an increasing number of deaths in patients on the lung transplantation waiting list.

The mismatch between the increasing number of potential receivers and the limited availability of lung grafts from brain-dead patients stresses the need for alternative resources for grafts. Among these, marginal donors recovering, lobar, split, or living-relative donor transplantation are being explored. However, one of the most promising ways to alleviate lung graft shortage would be to consider lung transplantation from donation after cardiac deaths (DACD) [3]. If only 2% of the deceased patients belonging to the Maastricht I and II categories [4] could become DACD, this would represent 7500 additional organ donors in Europe every year [5].

The expansion of the pool of potential donors by the inclusion of the DACD has proven to be very successful in kidney transplantation programs [6] in France. However, DACD lung transplantation has not been yet authorized by the French National Agency for Transplantation (Agence de la Biomédecine), but could be considered in the near future [7].

Lung preservation and pretransplantation functional assessment are the main medical issues in DACD procedures [3]. Lung is atypical among other recovered organs as lung parenchymal cells have low metabolic requirement and do not exclusively depend on perfusion for their survival, as respiration occurs via air spaces. The functional efficiency of the lung graft can be assessed by different means. As lungs are purposed to oxygenate venous blood and clear carbon dioxide (CO_2), blood gas analysis is definitely one of them. The assessment of the mitochondrial homeostasis may also be of paramount importance in the evaluation of the functional efficiency of the lung graft. Indeed, mitochondria play a key role in cellular energy metabolism as they are coupled to oxidative phosphorylation, responsible for adenosine triphosphate (ATP) synthesis and sustained lung viability. Last, induction of inflammatory reaction and innate immunity during ischemia and reperfusion of the graft represent an early trigger of acute allograft rejection. Indeed, activation of class I pattern recognition receptors (like receptor for advanced glycation endproducts, RAGE) in the donor organ contributes to inflammatory spread after graft reperfusion that can be assessed through cytokine levels [8].

Prehospital and in-hospital management of potential DACD are particularly challenging in France because of conflicting ethical and technical considerations regarding body integrity preservation, lung functional preservation, and graft assessment. Topical cooling is the historical technique used for lung preservation during cold ischemia and has been successfully translated from bench to clinical practice [9]. However, topical cooling has some drawbacks: it may injure the graft by requiring prolonged lung deflation [10]. It also requires a second step consisting in an *ex-vivo* lung perfusion system [11] to assess lung functionality before transplantation. Alternative techniques, allowing for lung inflation and providing equivalent preservation conditions to cold ischemia, but easier to implement in daily clinical practice are warranted in community hospitals where lung retrievals are performed.

Among these techniques, percutaneously inserted selective lung perfusion *in situ* appears particularly appealing. Indeed, it would allow simultaneous preservation, cooling, and assessment of the lung graft, it would not require an *ex-vivo* assessment rig, and would respect the deceased body's integrity. It would also be compatible with recovery of intra-abdominal organs requiring intra-aortic double balloon catheter and inferior vena cava vent. However, to the best of our knowledge, such a system has not been assessed yet.

This study aimed at assessing the functional preservation of the lung graft in an *in situ* anterograde selective lung perfusion performed in swine DACD model. We specifically focused on alveolo-capillary gas exchange, mitochondrial homeostasis, inflammation, and innate immunity to provide a wide spectrum of functional assessment. Percutaneously inserted selective lung perfusion *in situ* remains technically demanding and requires specifically designed cannula. Therefore, we first went for a feasibility study with open access to the heart chambers and checked whether lung preservation was actually achieved.

Materials and Methods

Animal preparation

Experimental work on animals conformed to the guidelines laid out in the Guide for the Care and Use of Laboratory Animals, provided by the French National Academy of Science and French Institute of Health guidelines for ethical animal research. The study was approved by the Institutional Review Board for the care of animal subjects (authorization number 67-147) and all animals received humane care in compliance with the European Convention on Animal Care. For ethical and effectiveness concerns, this study protocol was established and designed along with another study group focusing on the metabolomic consequences of

the pulmonary graft quality during cold ischemia [12]. This share was planned *a priori* to minimize the number of animal sacrificed in the different study groups.

After institutional ethics board approval, eight large white pigs (27 ± 4 kg) were studied under standard general anesthesia. Animals were fasted overnight in a thermo neutral environment with free access to water until the morning of the experiment. Before arrival in the operating room, they were premedicated with intramuscular ketamine (50 mg/kg) and azaperone (2 mg/kg). A 22-gauge peripheral venous line was inserted into an ear vein and general anesthesia was then induced with propofol (3 mg/kg). Depth of anesthesia was checked by paw pinch before muscular relaxation was administered. Endotracheal intubation (6 mm Portex® tube, Smiths Medical, UK) was facilitated with IV pancuronium (0.1 mg/kg). Mechanical ventilation was controlled with an Aisys® Carestation® (GE Healthcare™, Limonest, France) in conventional gas mixture (50% O_2, 50% N_2O) with a 2 l/min fresh gas flow. Tidal volume was set at 10–12 ml/kg and minute ventilation was adjusted to keep end-tidal carbon dioxide ($etCO_2$) between 35 and 45 mmHg, with a positive end-expiratory pressure of 5 cmH_2O. Anesthesia was maintained with 0.5% isoflurane.

Animal monitoring consisted of electrocardiography, pulse oximetry, capnography, airway pressure (peak, mean, and end-expiratory), mediastinal (intra-scissural), and rectal temperature, invasive blood pressure in the superficial femoral artery and pulmonary artery pressure with a balloon-tipped pulmonary artery catheter surgically inserted into the right internal jugular vein (Swan-Ganz CCOmbo® CCO, 7.5F pulmonary artery catheter, Edwards Lifesciences, Maurepas, France). As thermodilution cardiac output measurements are inaccurate during extracorporeal membrane oxygenator (ECMO) [13], Swan-Ganz catheter was not connected to a cardiac output monitor. As a result, we had no access to pulmonary blood flow and flow-derived variables until ECMO circuit was run (T_{30}). Data were continuously collected using Datex-Ohmeda S/5 Collect™ system (GE Healthcare, Limonest, France) plugged on the Aisys® Carestation®.

Surgical procedure

The time-line of the experimental protocol is depicted in Fig. 1. After a 30-min steady state time period during which hemodynamic variables remained in a 10% variation range, the surgical preparation was performed by a thoracic surgeon.

Heart and lungs were exposed via a median sternotomy and a pericardotomy. Superior and inferior vena cava, aortic arch, right, and left pulmonary artery were identified and encircled with a surgical line. Left and right ventricles were then cannulated with a 20-French and a 30-French cannula, respectively, (Medtronic®, Minneapolis, MN, USA) for further anterograde lung perfusion.

Cardiac arrest was induced by whole blood subtraction (40 ml/kg) via right ventricle cannulation followed by both aortic and caval clamping. The collected blood was then used for Steen Solution® preparation (Vitrolife™, Goteborg, Sweden). The ventilator was stopped and lungs were kept deflated. After 30 min of "warm ischemia" (no ventilation, no circulation), an anterograde selective pulmonary cold perfusion was started via a biventricular cannulation with aortic and caval clamping to induce "cold ischemia" (Fig. 2). The perfusion was performed using an ECMO (Primo2X®, Sorin Group™, Milan, Italy) perfused with a cooled (4 °C) organ preservation solution (Perfadex®, Vitrolife™) whose temperature was continuously controlled (SARNS TCM II, 3M Health Care, Ann Arbor, MI, USA). Simultaneously, pulmonary ventilation was resumed without changing the ventilator settings.

To challenge the oxygen and carbon dioxide diffusion through the alveolo-capillary membrane, a deoxygenated, carboxylated, and warmed (22 °C) blood substitute containing red blood cells (Steen Solution®, Vitrolife™) was perfused for a 30-min period at 180, 360, and 480 min after cardiac arrest. Systemic oxygen consumption and carbon dioxide production were mimicked by filling the ECMO with a hypoxic gas (N_2: 86%, O_2: 7%, CO_2: 7%). This intermittent cold perfusion with Perfadex® followed by warm

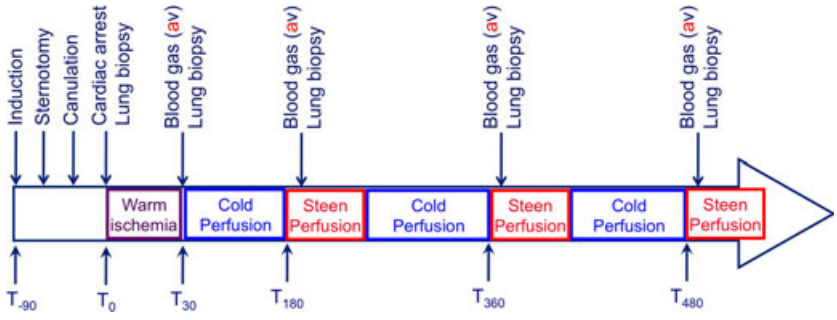

Figure 1 Time-line of the experimental protocol.

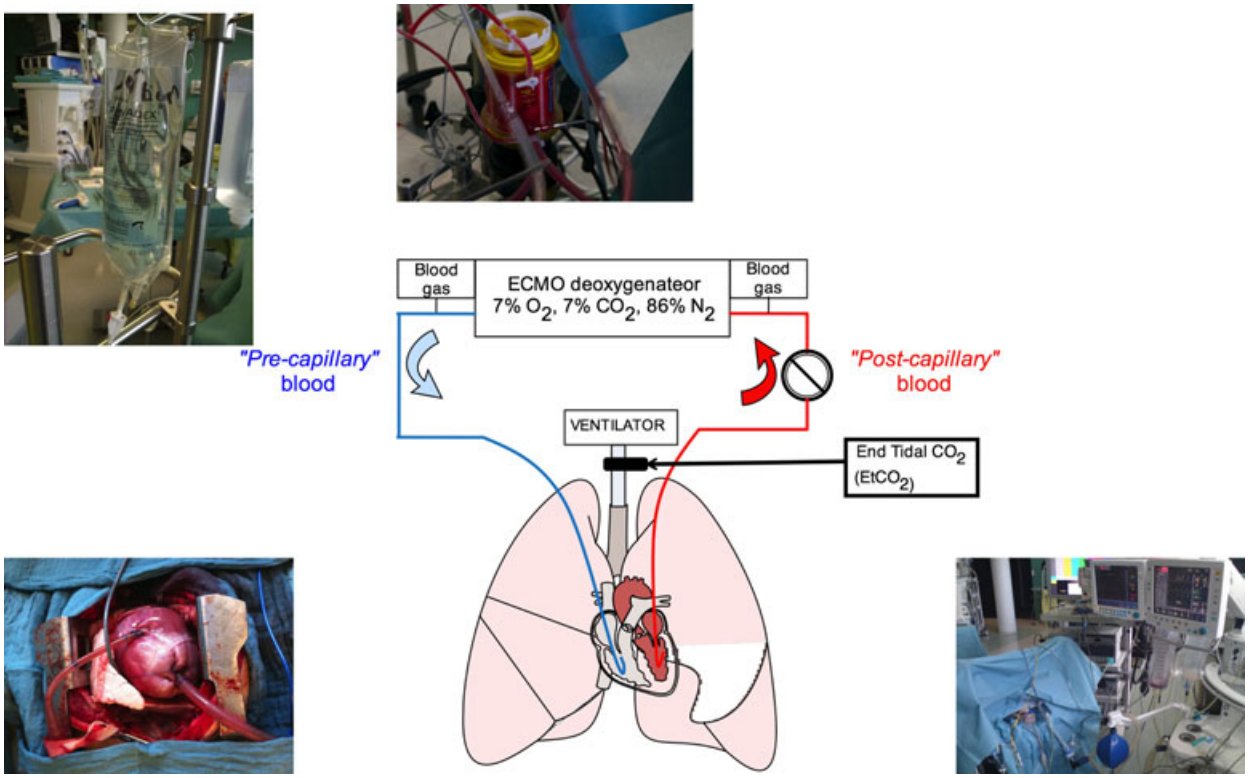

Figure 2 *In situ* anterograde selective lung perfusion model.

perfusion with Steen Solution® allowed us to intermittently evaluate functional graft performance and to challenge the lungs with blood perfusion.

Pulmonary hemodynamics and gas exchange assessment

Mean pulmonary arterial pressure was measured at T_0, T_{180}, T_{360}, and T_{480} time points and total pulmonary vascular resistance (PVR) was estimated (at T_{180}, T_{360}, and T_{480}) using the following formula:

$\text{PVR}[\text{dynes.s.cm}^{-5}] = \frac{\text{mPAP}\times 79.9}{Q}$ with mPAP representing mean pulmonary artery pressure (mmHg) and Q the ECMO output (l/min) [14]. As we did not measure left arterial pressure or its surrogate (pulmonary capillary wedge pressure), exact calculation of PVR could not be performed.

Once a 22 °C core temperature was reached, blood gas analysis (Rapidlab 865®, Siemens, Erlangen, Germany) was performed on the venous and arterial line of the ECMO.

This allowed the calculation of the PaO_2/FiO_2 ratio representing the ratio of postcapillary oxygen partial pressure (mmHg) over the inspired oxygen fraction (%).

Oxygen diffusion was then calculated using the arteriovenous oxygen content difference ($C(a\text{-}v)O_2$) and the transpulmonary oxygen output ($tpVO_2$):

$$C(a\text{-}v)O_2 = 1.34 \times Hb \times (SaO_2 - SvO_2) + 0.003 \times (PaO_2 - PvO_2)$$

where Hb is the hemoglobin concentration (g/dl), SaO_2 the postcapillary arterial saturation (%), SvO_2 the precapillary venous saturation (%), PaO_2 the postcapillary oxygen partial pressure (mmHg), and PvO_2 the precapillary oxygen partial pressure (mmHg).

$$tpVO_2 = 10 \times C(a\text{-}v)O_2 \times Q$$
$$[Q : \text{ECMO output (l/min)}]$$

Carbon dioxide diffusion through the alveolo-capillary membrane was assessed using the end-tidal carbon dioxide partial pressure ($etCO_2$) out the endotracheal tube and the calculated transpulmonary CO_2 clearance ($tpCO_2$):

$$tpCO_2 = 10 \times (PvCO_2 - PaCO_2) \times Q$$
$$[Q : \text{ECMO output (l/min)}]$$

where $PaCO_2$ is the postcapillary carbon dioxide partial pressure and $PvCO_2$ the precapillary carbon dioxide partial pressure. As CO_2 content is linearly related to CO_2 tension over the physiologic range of CO_2 contents [15,16], the

difference between precapillary and postcapillary carbon dioxide pressure ($PvCO - PaCO_2$) was used as a surrogate of arteriovenous CO_2 content difference.

$tpVO_2$ and $tpCO_2$ are flow-dependent variables (either cardiac or ECMO output). As we had no access to cardiac output before ECMO circuit was run, $tpVO_2$ and $tpCO_2$ were only available at T_{180}, T_{360}, and T_{480} time points.

Pulmonary mitochondrial respiratory rate

Lung mitochondria isolation

Preparation of mitochondria was adapted from a previously described procedure [17]. After recovering from peripheral biopsy, lung tissue (including parenchymal, vascular, and inflammatory cells) was placed in buffer A containing 50 mM TRIS, 1 mM EGTA, 70 mM sucrose, 210 mM mannitol with pH adjusted to 7.4 at 4 °C. Tissues were then finely minced with scissors and homogenized using a Potter Elvejem. Homogenate was then centrifuged during 3 min at 1300 *g* and 4 °C. The supernatant was centrifuged at 10 000 *g* for 10 min, 4 °C to sediment mitochondria. Finally, the mitochondrial pellet was washed twice and then suspended with buffer B containing 50 mM TRIS, 70 mM sucrose, 210 mM mannitol with pH adjusted to 7.4 at 4 °C. Protein content was routinely assayed using bovine serum albumin as a standard (Gornall's procedure, Roti®-Quant universal, Carl Roth GmbH, Karlsruhe, Germany). Mitochondria were kept on ice and used within 4 h.

Lung mitochondrial respiratory function

Lung maximal oxidative capacity and the relative contribution of the respiratory chain complexes I, II, III, and IV to the global mitochondrial respiratory rate were studied in isolated mitochondria in buffer M containing 100 mM KCl, 50 mM 3-(N-morpholino) propanesulfonic acid (MOPS), 1mM EGTA, and 5 mM Kpi at 25 °C. Oxygen consumption was measured polarographically using a Clark-type electrode (Strathkelvin Instruments, Glasgow, Scotland), as previously described [18–20].

When maximal, adenosine diphosphate (ADP)-stimulated fiber respiration (V_{max}) was recorded, electron flow went through complexes I, III, and IV, because of the presence of glutamate (5 mM) and malate (2 mM). Complex I was blocked with amytal (0.02 mM) and complex II was stimulated with succinate (25 mM). Mitochondrial respiration in these conditions allowed determining complexes II, III, and IV activities (V_{succ}). After that, N,N,N′,N′-tetramethyl-p-phenylenediamine dihydrochloride (TMPD, 0.5 mM) and ascorbate (0.5 mM) were added as an artificial electron donor to complex IV. Complex IV activity was then determined as an isolated step of the respiratory chain ($V_{TMPD/Asc}$). Respiration rates were expressed as μmol O_2/min/g protein. The coupling of phosphorylation to oxidation was determined by calculating the ACR as the ratio between
ADP-stimulated respiration (V_{max}) and basal respiration (without ADP) with glutamate and malate as substrate (V_0) [21].

Lung biopsy time course

Tissular lung biopsy was performed to assess the mitochondrial respiratory rate at the initiation of cardiac arrest (T_0), at the end of the 30-min warm ischemia (T_{30}), 3 h (T_{180}), 6 h (T_{360}), and 8 h (T_{480}) after the beginning of cold ischemia (Fig. 1). Simultaneously, bronchoalveolar lavage fluid (BALF) was obtained via fiberoptic bronchoscopy.

Inflammation and induction of innate immunity in the lung graft

The secreted protein levels of RAGE, interleukine-6 (IL-6), and tumor necrosis factor alpha (TNF-α) were determined in bronchoalveolar lavage (BAL) supernatants with the respective commercial ELISA kits (MyBiosource, R&D Systems, and Thermo Scientific, respectively). All samples were tested in triplicate and read at 450 nm using an ELISA plate reader (VersaMax, Molecular Devices).

Statistical analysis

Results are expressed as means ± SEM, compared using repeated measures analysis of variance (ANOVA) and Newman–Keuls *post hoc* test with the GraphPad™ Prism® software. Statistical significance was set at the 0.05 level.

Results

Systemic monitoring

Hemodynamic variables

Of the eight included animals, the first two were used to validate the experimental procedure and 6 pigs were finally included in the final analysis.

Systolic (SBP) and diastolic (DBP) blood pressure significantly dropped to mean systemic pressure after cardiac arrest (SBP = 10.9 ± 3.8 mmHg; DBP = 10.2 ± 3.8 mmHg) compared to induction (SBP = 73.4 ± 4.8 mmHg; DBP = 39.4 ± 1.6 mmHg, $P < 0.05$) and sternotomy values (SBP = 84.7 ± 6.3 mmHg; DBP = 45.7 ± 2.6 mmHg, $P < 0.05$).

Neither mPAP ($P = 0.69$, Fig. 3a) nor PVR ($P = 0.46$, Fig. 3b) was significantly altered during the 8-h perfusion period.

Mediastinal temperature

Mediastinal temperature was 35.7 ± 1.1 °C at sternotomy. After cardiac arrest during cold ischemia, mediastinal temperature was below the range of the probe (<10 °C) and

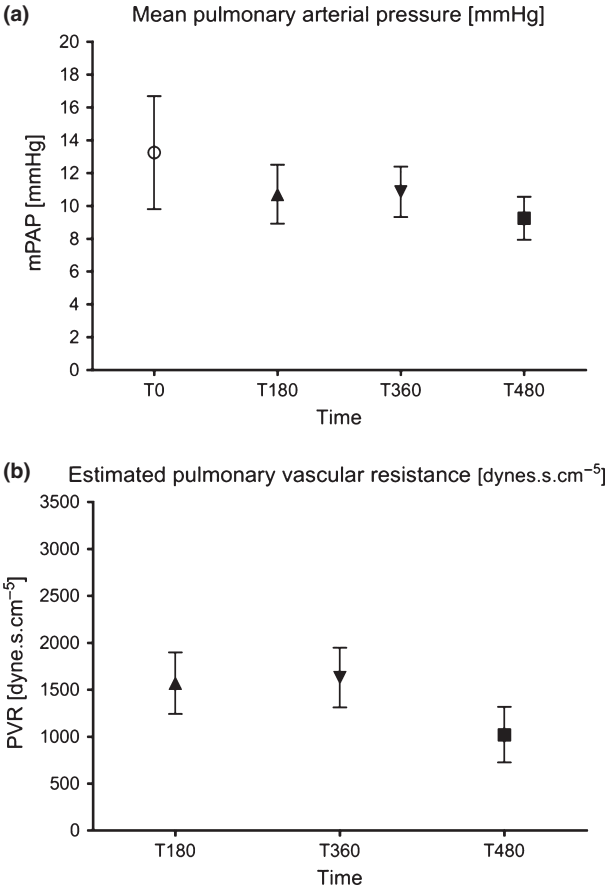

Figure 3 Time course of mean pulmonary arterial pressure (mPAP, a) and pulmonary vascular resistance (PVR, b) throughout the protocol.

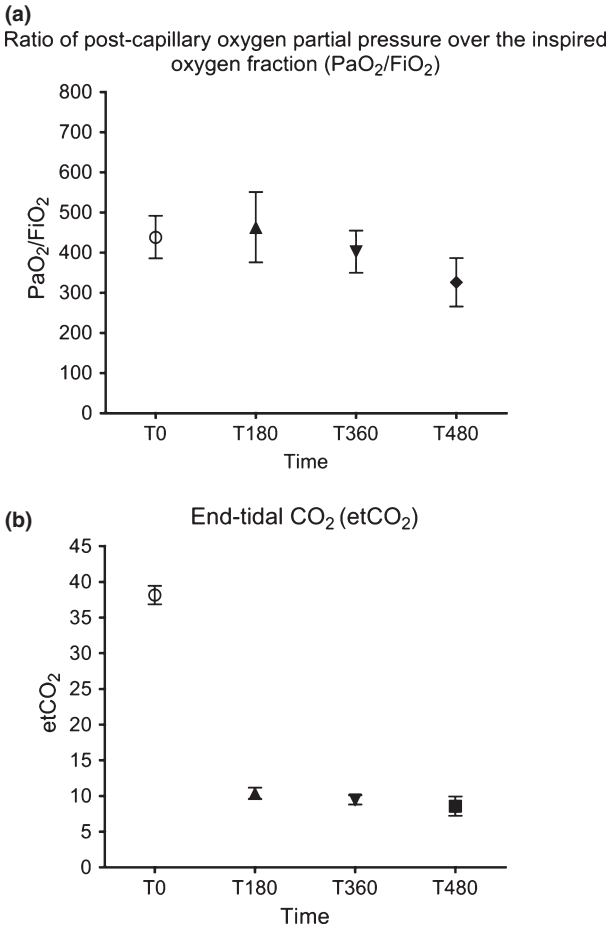

Figure 4 Time course of ratio of postcapillary oxygen partial pressure over the inspired oxygen fraction (PaO_2/FiO_2 ratio, a) and end-tidal carbon dioxide partial pressure ($etCO_2$, b) throughout the protocol.

reached 25.6 ± 4.3 °C, 26.8 ± 2.0 °C, and 27.7 ± 1.5 °C during 3, 6, and 8 h Steen Solution® perfusion, respectively.

Pulmonary gas exchange evaluation

Flow-independent variables

The PaO_2/FiO_2 ratio was maintained above 400 from cardiac arrest to 360 min and slightly declined thereafter (Fig. 4a). However, taking the four time points, global change was nonsignificant (ANOVA $P = 0.56$).

Flow-dependent variables

End-tidal CO_2 abruptly dropped after cardiac arrest, but eventually reached the same value (10.5 ± 1.0 mmHg; 9.5 ± 0.7 mmHg and 8.1 ± 1.8 mmHg) during the Steen perfusion® challenges at T_{180}, T_{360}, and T_{480}, respectively (Fig. 4b; ANOVA $P = 0.41$ for these three time points).

Arteriovenous O_2 content difference (Fig. 5a), transpulmonary O_2 output (Fig. 5b), and transpulmonary CO_2 clearance (Fig. 5c) did not change significantly between T_{180} and T_{480} (ANOVA $P = 0.83$, $P = 0.46$, $P = 0.24$, respectively).

Lung mitochondrial respiratory function

Table 1 displays baseline values and the kinetic of the lung mitochondrial respiratory rate at T_0, T_{30}, T_{180}, T_{360}, and T_{480} after cardiac arrest.

Importantly, maximal lung mitochondrial respiration (V_{max}), complexes II, III, and IV activities (V_{succ}) and complex IV activity ($V_{TMPD/Asc}$) were not significantly altered up to 8 h after cardiac arrest. The ACR, reflecting the coupling of phosphorylation to oxidation, also remained unaltered during cold perfusion and showed trending values similar to $tpVO_2$ and $tpCO_2$.

Inflammation and innate immunity in the lung graft

IL-6 and TNFα assays

Interleukine-6 and TNFα concentrations in BALF were under the threshold of detection (set at 18.8 and 31.3 pg/ml by the manufacturer, respectively) at any time, suggesting

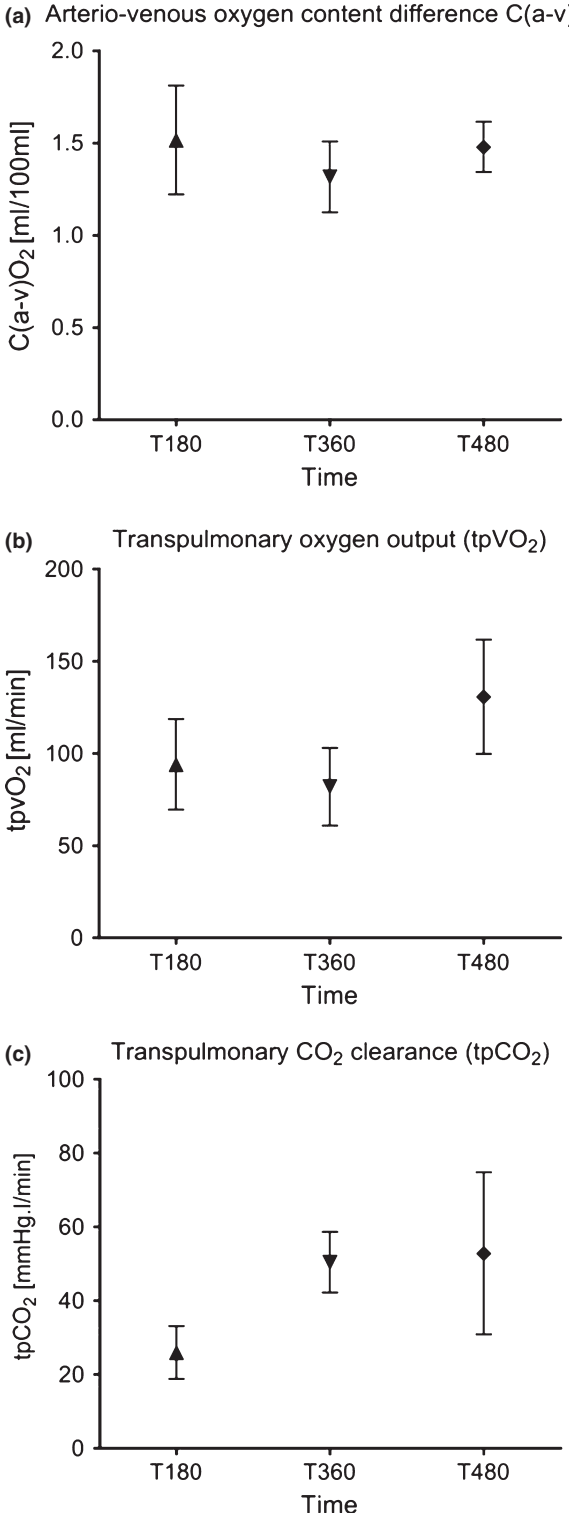

Figure 5 Time course of the arteriovenous oxygen content difference ($C(a\text{-}v)O_2$, a), transpulmonary oxygen output ($tpVO_2$, b), and transpulmonary CO_2 clearance ($tpCO_2$, c) throughout the protocol.

very low or absence of inflammation in the lungs under cold ischemia.

Receptor for advanced glycation endproducts assay

RAGE concentrations in BALF were low at baseline and were not altered over time in the lungs under cold ischemia ($P = 0.24$), Fig. 6.

Discussion

Sustained oxygen and carbon dioxide diffusion through the alveolo-capillary membrane, preserved mitochondrial homeostasis, and absence of inflammation in the lung tissue up to 8 h after cardiac arrest suggest a long-lasting functional preservation of the lung graft in this large animal DACD model with our new anterograde perfusion system.

Our protocol consisted in a repetition of intermittent cold perfusion with Perfadex® followed by short warm perfusion bouts with Steen Solution® to intermittently challenge the alveolo-capillary membrane in "pseudo-physiologic" conditions (warm blood substitute). Indeed, compared with static cold storage, hypothermic machine perfusion was shown to better preserve lung compliance and pulmonary oxygenation and to decrease both PVR and oxidative damage during reperfusion [22]. Besides the conventional PaO_2/FiO_2 ratio, we also incorporated multiple flow-derived variables like $tpVO_2$ and $tpCO_2$, which describe the graft ability to oxygenate and decarboxylate a given amount of blood flowing through the lung vasculature.

In addition to blood gas analysis, we used mitochondrial function assessment as a surrogate of ischemic graft viability in this animal DACD model. Indeed, one of the main concerns in DACD protocols is the difficulty in assessing the viability of the ischemic lung tissue after cardiac arrest. Surrogates of lung cell homeostasis include total adenine nucleotides and ATP. However, their assay requires overnight lyophilization and is not always compatible with the challenging time lines for organ procurement and assessment [23,24]. In the lung, ATP is mainly produced by pulmonary mitochondria and mitochondrial failure in the lung was previously demonstrated to be linked to ATP breakdown [25]. In the case of prolonged ischemia, metabolites and reactive oxygen species (ROS) accumulate within the cell and promote mitochondrial dysfunction at reperfusion, leading eventually to apoptosis and necrosis. This represents the ultimate spectrum of the ischemia-reperfusion (IR) injury. Conversely, if perfusion can be timely restored before ischemia reaches a critical duration, mitochondrial function can recover and allow for cell survival.

As far as IR injury mechanisms are concerned, most studies have pointed out to the mitochondria as an early target and the central player in cell survival. Indeed,

Table 1. Lung mitochondrial respiratory chain complexes activities and coupling of phosphorylation to oxidation.

	T_0	T_{30}	T_{180}	T_{360}	T_{480}	ANOVA *P*-value
V_{max}	21.3 ± 3.3	23.4 ± 3.2	16.4 ± 3.8	19.4 ± 1.1	24 ± 2.4	0.42
V_{succ}	17.8 ± 2.9	19.8 ± 1.6	15 ± 2.7	15.4 ± 0.8	18.0 ± 2.0	0.50
$V_{TMPD/Asc}$	28.9 ± 6.9	26.5 ± 4.3	23.9 ± 5.0	23.3 ± 2.6	25.9 ± 4.0	0.93
ACR	13.8 ± 5.7	15.6 ± 8.3	9.6 ± 0.9	8.0 ± 1.2	14.0 ± 1.9	0.8

Data are displayed before (T_0) and 30, 180, 360, and 480 min after cardiac arrest (T_{30}, T_{180}, T_{360}, T_{480}). Results are expressed as mean ± SEM. V_{max}, complexes I, III, IV activities, using glutamate and malate; V_{succ}, complexes II, III, IV activities, using succinate; $V_{TMPD/asc}$, complex IV activity using TMPD/Ascorbate, as mitochondrial substrates. Respiration rates are expressed as μmol O_2/min/g protein; ACR, acceptor control ratio.

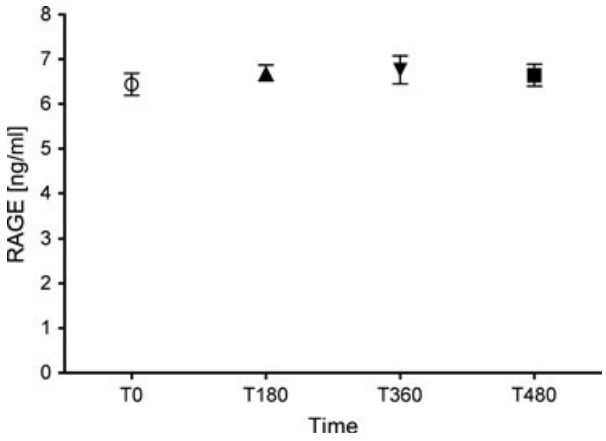

Figure 6 Time course of receptor for advanced glycation endproducts (RAGE) concentrations in bronchoalveolar lavage fluid throughout the protocol.

mitochondria are the source of ATP storage, the main core of ROS generation through electron leakage across the mitochondrial respiratory chain [26] and the mainstay of apoptosis regulation. The ACR (reflecting mitochondrial oxidative phosphorylation) seems to be an interesting surrogate for cell viability. As a matter of fact, Hirata *et al.* demonstrated that it could be assessed within 1 h and made it possible to ascertain the condition of the ischemic lung before performing the recipient anesthesia in a clinical lung transplantation setting [27]. It was also a sensitive marker, showing significant elevation 1 h after cardiac arrest, when lactate levels in the lungs exhibited no significant change. In cultured human endothelial cells, Stadlmann *et al.* demonstrated that 8 h of simulated cold IR reduced complexes I, II, and IV respiration pointing to a general mitochondrial defect [28]. This is in contrast to our experiments in which ACR, as well as V_{max}, V_{succ}, and $V_{TMPD/Asc}$ remained unaltered during the whole procedure. Interestingly, whatever the substrates used and therefore the complexes studied, no deleterious effects on the mitochondrial respiratory function were observed. This is in line with the blood gas analysis of lung graft and further supports the efficacy of our selective *in situ* anterograde perfusion system.

Concerning inflammation, our results shown for IL-6, TNFα, and RAGE measurements are in line with an absence of inflammation and innate immunity induction. Usually, during solid organ transplantation, IR-induced damaged associated molecular patterns are recognized by receptors for advanced glycation endproducts, which amplify full-scale lung IR injury. They also convert immature dendritic cells to mature ones that translate innate to adaptive immunity [8]. In addition, activation of the RAGE pathway is involved in increased endothelial permeability [29] and blockade of RAGE attenuates pulmonary reperfusion injury in mice [30]. Moreover, elevated levels of RAGE in the alveolar fluid predict reduced alveolar fluid clearance in isolated perfused human lungs [31] and RAGE levels are significantly higher in donor lungs that subsequently develop sustained primary graft dysfunction (PGD) versus transplanted lungs that do not display PGD [32]. As RAGE levels in BALF did not increase over time, selective pulmonary lung perfusion does not seem to activate the deleterious consequences of the RAGE pathway. Cytokine expression in lung graft before implantation also has a strong predictive value for PGD and mortality. Indeed, Kaneda *et al.* demonstrated that IL-6, IL-8, TNFα, and IL-1β were risk factors for mortality, and IL-10 and IFN-γ were protective factors [33]. In the recipient, Moreno *et al.* showed that there was a significant elevation of IL-6 in blood and BAL during the first few hours after reperfusion of the graft, which was directly related to the development of PGD [34]. This was clearly not the case during our anterograde perfusion of the lungs.

Historically, the first cold ischemia lung preservation consisted of topical cooling [35] and lung functional preservation has been assessed up to 6 h with this technique [36]. However, topical cooling may prove technically challenging: it requires four drains, inflow and outflow tracts, continuous roller pump, and 6 l ice-cold saline. With longer intervals of topical cooling exceeding 6 h, clots in the pulmonary artery become organized and difficult to remove resulting in poor outcome in the isolated

reperfusion model [36]. Sustained ventilation and topical cooling cannot be applied simultaneously because topical cooling requires lung deflation and atelectasis. The extent of lung inflation during the ischemic period affects the severity of lung injury and if the lungs are kept deflated during the ischemic period, the resulting injury is major, including inflammation, edema, and reduced blood flow [37–39]. As a result, topical cooling may induce graft injury.

Pulmonary perfusion with cold solution is an emerging way of cold ischemia graft preservation and could perform even better. In a study comparing topical cooling with single-shot *in situ* flush perfusion (both anterograde and retrograde) after ventilator switch-off, Erasmus *et al.* demonstrated that flush perfusion provided lower alveolar-arterial oxygen gradient, decreased ventilation pressure, and lung edema compared with topical cooling in pigs [40]. As our study protocol did not allow us to simultaneously compare topical cooling and *in situ* anterograde lung perfusion, we could not confirm these findings, but plan to compare selective lung perfusion with standard ice storage and topical cooling in further studies, the ultimate step being the transplantation of the perfused lungs.

Although our preliminary results are promising, this study suffers from some limitations to translate our experimental protocol to the clinical DACD situation. First, induction of cardiac arrest was performed through exsanguination followed by clamping of aorta and caval veins. As a result, the agonal phase prior to cardiac death was rather short and may not have been long enough to induce the inflammatory reaction encountered in the clinical scenario of a dying patient experiencing hypoxemia [41] and long-lasting hypotension [42]. The duration of warm ischemia was restricted to 30 min because, on a bioenergetic basis, swine lungs submitted to warm ischemia can be suitable for transplantation if the warm ischemia duration does not exceed 30 min [43]. Second, we lacked a control group in which explanted lungs would have been maintained on ice after an initial cold flush.

Third, this first experiment in swine was a feasibility study with open surgical access preceding total percutaneous cannulation with dedicated cannulae. One of these cannulae, percutaneously inserted via the right internal jugular vein, would include two external inflatable balloons to occlude the superior and inferior vena cavae without reaching the suprahepatic veins. The other one, percutaneously inserted via the right carotid artery, would occlude the ascending aorta and vent the left ventricular outflow tract, avoiding congestion of intra-abdominal organs. Future experiments with percutaneous access with specifically designed cannula are the next step in our DACD protocol. If these experiments turned out to be successful, *in situ* lung perfusion would provide time for the lung recovery team to perform a comprehensive lung assessment in the clinical arena and extend the delay after cardiac arrest.

Authorship

JP and EN: performed research, collected data, analyzed data, and wrote the paper. NS: designed research, performed research, collected data, analyzed data, and wrote the paper. A-LC: performed research contributed important reagents, collected data, analyzed data. MB: performed research and collected data. MC: performed research and contributed important reagents. NF and IJN: contributed important reagents. BG: contributed important reagents, analyzed data, and wrote the paper. GM: designed research, analyzed data, and wrote the paper. PD: performed research, analyzed data, and wrote the paper.

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